DISTINCT MICROTUBULE SUBSETS ORGANIZE AROUND THE NEWLY FORMING LUMEN DURING EPITHELIAL POLARIZATION

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Epithelial tissues maintain body homeostasis by acting as a barrier to the outside environment and allowing for the vectorial transport of molecules and solutes. To establish this barrier, epithelial cells must form and maintain distinct apical and basolateral domains separated by tight junctions. This polarization process is triggered by signals initiated at cell-substratum and cell-cell adhesions and propagated by rho GTPases and multiple signaling complexes. Cytoskeletal reorganization accompanies polarization and both microtubules and kinesin-mediated transport are required for apical membrane maintenance. However, how microtubules respond to polarization cues and affect formation of the apical membrane is currently unknown. To address this, I characterized how microtubules reorganize during early events in epithelial polarization. I utilized both 2D and 3D cell culture to visualize microtubule subsets during early polarization events and examined the effects of perturbing microtubule dynamics on lumen formation. Two biochemically distinct subsets of microtubules reorganized around the vacuolar apical compartment (VAC), a transient organelle containing apical proteins, and again following exocytosis into the lumen. Dynamic microtubules arranged perpendicular to VACs and lumens, and post-translationally modified, stable microtubules arranged circumferentially around VACs and against the lumen face. Nocodazole-induced microtubule depolymerization and taxol-induced microtubule stabilization either delayed or halted lumen formation in both systems. Microtubule regrowth revealed that microtubules are nucleated near

the VAC/lumen and at the cell periphery while live recordings of fluorescently tagged EB1 revealed that growing microtubule plus ends are oriented both towards and away from the nascent apical membrane. These findings show that microtubules are required for lumen formation and rearrange as the nascent apical membrane is transcytosed to form a lumen. Aspects of the microtubule array are indicative of how microtubules might respond to polarization cues and how kinesins might recognize distinct tracks for targeted transport. Since adherens junctions can induce microtubule stabilization and recruit dynein, it is likely that microtubules are stabilized and modified following cell-cell contact. Then dynein might provide a pulling force on stabilized MTs to reposition VACs prior to lumen formation. Finally, kinesins might use MT modifications to direct apical membrane exocytosis at the lateral membrane to form a lumen.

BIOGRAPHICAL SKETCH

William Oliver Smith was born in Lodi, California. He attended the University of the Pacific in Stockton, California, majoring in biochemistry. He graduated Summa Cum Laude with honors, obtaining a BS in Biochemistry with two minors in Mathematics and Technology in May of 2009. He moved to New York City, New York in August of 2009 to study for a Ph.D at Weill Cornell Graduate School of Medical Sciences, where he joined Dr. Geri Kreitzer's lab in April of 2010. During his time at Weill Cornell, he served in many positions within student government and organized events for the American Society of Cell Biology. William is the second person in his family to obtain a doctorate, the first being his grandfather and namesake, Reverend William Oliver Smith.

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"There are moments when one has to choose between living one's own life fully, entirely, completely- or dragging out some false, shallow, degrading existence that the world in its hypocrisy demands." – Oscar Wilde

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LIST OF ABBREVIATIONS

+TIP: plus end tracking protein

AMIS: Apical membrane initiation site

AMP: Adenosine monophosphate

ATP: Adenosine triphosphate

ddH_2O: Double distilled water

DMEM: Dulbecco's modification of Eagle's medium

DNA: Deoxyribonucleic acid

ECM: Extracellular matrix

F-actin: Filamentous actin

G-actin: Globular actin

GAP: GTPase activating protein

GDP: Guanosine diphosphate

GEF: Guanine nucleotide exchange factor

GFP: Green fluorescent protein

Glu MT: Detyrosinated microtubule

GPI: Glycophosphatidyl inositol

GTP: Guanosine triphosphate

IFT: Intraflagellar transport

MAP: Microtubule associated protein

MDCK: Madin Darby canine kidney

mRNA: Messager ribonucleic acid

MTs: Microtubules

ON: Overnight

PBS: Phosphate buffered saline

PI(3,4,5)P2: Phosphatidylinositol 3,4,5-trisphosphate

PI(4,5)P2: Phosphatidylinositol 4,5-bisphosphate

PTM: Post-translational modification

RT: Room temperature

SMEM: Spinner modification of Eagle’s medium

TGN: Trans Golgi network

Tyr MT: Tyrosinated microtubule

VAC: Vacuolar apical compartment

CHAPTER ONE

INTRODUCTION

Epithelial tissue function and consequences of dysfunction

Epithelial and epithelial-derived tissues comprise 60% of the human body and line external organ surfaces, acting as a barrier to the outside environment and in maintining physiological homeostasis (Bryant and Mostov 2008). To establish this barrier, epithelial cells polarize the cell membrane and form impermeable junctions between contacting cells. This allows for vectorial transport of molecules and solutes through the membrane to absorb nutrients, eliminate wastes, and maintain an ionic and osmotic balance. Cell-cell junctions separate the luminal-facing apical domain from the cell-contacting lateral domain and basement membrane-contacting basal domain, often described together as the basolateral domain due to their similar lipid and protein composition. In vertebrates, tight junctions prevent the diffusion of transmembrane proteins and lipids between these domains, which keeps membrane compartments distinct and maintains their specialized functions. A number of diseases are linked to polarization defects in epithelial tissues, such as polycystic kidney disease, ciliopathies, and epithelial cancers (Mellman and Nelson 2008). This is often due to missorting of proteins that either maintain tissue function, promote cell proliferation and migration, or keep cells in the proper orientation. Studying how epithelial cells polarize not only aids in our understanding of tissue development and organ function, but is vital for elucidating the mechanistic basis of disease and in identifying new modes to treat human pathologies. This thesis will discuss the mechanisms in place to establish and maintain epithelial polarity, identify unanswered questions in the

field, and draw correlations from other polarizing cell types and organisms to postulate potential answers to these questions.

Cell polarization and cytoskeletal reorganization

Almost all cell types in the body transition during differentiation from a symmetrical, non-polarized morphology to a polarized morphology, defined by the asymmetric distribution of lipids, proteins, and organelles within the cell. To achieve this asymmetry, cells utilize internal and external cues to direct membranes and proteins to different domains in the cell (Li and Gundersen 2008). One of the key events in breaking cellular symmetry is the reorganization of the actin and microtubule cytoskeleton, inherently polarized protein polymers that direct cell shape changes, signaling events, membrane trafficking, and organelle positioning.

Actin filaments (F-actin) are formed by polymerization of globular subunits (G-actin) that bind and hydrolyze ATP. F-actin assembly is activated by actin-nucleation factors, such as formins and the actin-related protein 2/3 (Arp2/3) complex, which can also nucleate actin branches on existing filaments (Pollard 2007). G-actin is added in the same orientation to produce a polarized filament with two distinct ends termed the barbed end, which adds ATP-bound subunits until capped, and pointed end, which has an equal on-off rate for G-actin subunits. Additional actin binding proteins contribute to the regulation of actin dynamics and organization by acting as either stabilizing or destabilizing factors (Stossel, Fenteany et al. 2006). Together with actin nucleators, these proteins act as the effectors of signaling events that control localized actin dynamics to stimulate growth, branching, severing, and bundling of fibers.

Microtubules (MTs) are comprised of heterodimers of α- and β-tubulin that bind GTP and assemble head-to-tail and laterally to form hollow tubes, giving them an inherent polarity denoted by a plus end and minus end (Amos and Klug 1974). GTP hydrolysis induces a structural change in the heterodimer that promotes disassembly of the MT lattice (Vale, Coppin et al. 1994). In undifferentiated cell types, MTs form a centrally nucleated radial array, with minus ends anchored at the MT organizing center (MTOC), often near the centrosome, and plus ends oriented out towards the periphery. MT plus ends undergo rounds of growth and shrinkage while anchored minus ends are kept static. This process, termed dynamic instability, allows MT arrays to rapidly reorganize within the cell and probe the cell periphery (Mitchison and Kirschner 1984). Polarization signals can induce changes in MT dynamicity, promote MT bundling, or stimulate capture of MT plus ends at the cell cortex to stabilize MTs (Gundersen and Bulinski 1988; Gundersen, Khawaja et al. 1989; Kollins, Bell et al. 2009). These changes are mediated by a number of proteins, including structural MT associated proteins (MAPs), which bind to the side of the MT lattice to hinder depolymerization and bundle MTs, and plus-end tracking proteins (+TIPs), proteins that associate with the MT plus end and promote growth (Jaworski, Hoogenraad et al. 2008). A variety of post-translational modifications accumulate on tubulin subunits in stable, long-lived MTs, resulting in biochemically distinct subsets of MTs. Long-lived MTs accumulate these modifications by the action of modifying enzymes that interact preferentially with MT polymer. Post-translational modifications on MTs are subsequently reversed by demodifying enzymes that interact preferentially with soluble tubulin dimers when they are released from depolymerizing MTs (Song and Brady 2015). While PTMs do not induce MT

stability, they can recruit MAPs to promote longevity and are hypothesized to distinguish tracks for kinesin-mediated membrane transport (Verhey and Gaertig 2007). These changes induce MTs to form more complex arrays and often lead to the creation of non-centrosomal nucleation and anchoring sites for MT minus-ends in the cell (Bartolini and Gundersen 2006).

Cytoskeletal motor proteins are key proteins that interpret and mediate cytoskeletal reorganization (Vale 2003). Three families of motor proteins, myosin, kinesin, and dynein, use ATP to generate force for directed movement along F-actin (myosin) or MTs (kinesin, dynein). This allows them to transport cargoes, such as proteins, vesicles, organelles, and mRNAs, to different places in the cell and alter the cytoskeleton. Most myosin family members move towards the barbed end of actin and can generate contractile forces by sliding actin filaments in opposite directions (Sellers 2000; Hartman and Spudich 2012). Most kinesin family members move towards the MT plus end and certain subfamilies have known roles in MT depolymerization, sliding, transport, and stabilization, while dynein moves towards the minus end and can generate pulling forces on MTs to reorient the centrosome (Hirokawa and Noda 2008; Drummond 2011; Walczak, Gayek et al. 2013). How the cytoskeleton reorganizes in response to polarization cues and how this affects polarization has been characterized in many different model systems. In the following sections, I will highlight well known polarity regulators and the cytoskeletal reorganization events they trigger in other systems before examining signaling events that mediate epithelial polarization.

Par complexes and rho GTPases: master regulators of polarity

Par complexes are master regulators of cell polarity in many different model systems. These complexes were first characterized from a set of

partitioning defect mutants in *C. elegans* that require proper function for anterior-posterior patterning in the egg prior to cell division (St Johnston and Ahringer 2010). Sperm fertilization provides a posterior polarity cue that induces a wave of actin-myosin contractions towards the anterior pole to initially separate par proteins at the cell cortex. Par3 and Par6 interact with cortical actin to localize to the anterior cortex of the egg while Par1 and Par2 localize to the posterior cortex. Each complex mutually antagonizes the other by promoting dissociation from the cortex, which maintains the two separate domains. The two other par proteins are Par4, which localizes to the entire cell cortex, and Par5, which remains cytoplasmic. These protein complexes are required to activate G protein complexes, which recruit dynein to pull on astral MTs and reposition the centrosome. This orients the mitotic spindle and induces an asymmetric protein distribution following the first rounds of cell division. Par3 and Par6 are scaffolding proteins that recruit atypical protein kinase C (aPKC) to form a conserved complex required for many polarization processes, including axonal specification in neurons and directed cell migration, while Par4 and Par1 are kinases that have known roles in MT reorganization, as discussed below (Goldstein and Macara 2007).

Three rho family small GTPases, Cdc42, RhoA, and Rac1, are master regulators of polarity that have well characterized roles in actin reorganization (Jaffe and Hall 2005). Small GTPases function as molecular switches and are activated by binding to GTP, promoted by guanine nucleotide exchange factors (GEFs), and inactivated following GTP hydrolysis to GDP, promoted by GTPase activating proteins (GAPs). In budding yeast, Rho activation stimulates formins to nucleate actin growth while Cdc42 localizes to the growing bud and binds the formin Bni1 to orient actin cables for directed,

polarized delivery of cargoes (Evangelista, Blundell et al. 1997). In migrating cells of metazoans, which polarize to form a protrusive leading edge and a contractile trailing edge, Cdc42 and Rac1 reorganize actin at the leading edge, where Cdc42 activates formins to produce actin bundles for protrusive filopodia while Rac1 activates Arp2/3 to produce branched actin for a wide, protrusive lamellipodia (Sadok and Marshall 2014). RhoA signals actin-myosin contraction along actin bundles for trailing edge contraction. In neurons, Cdc42 and Rac1 localize to the axonal growth cone and control the protrusive cortical actin network in a similar manner (Gonzalez-Billault, Munoz-Llancao et al. 2012).

Rho GTPases and par proteins both influence MT dynamics and reorganization by activating downstream factors (Wojnacki, Quassollo et al. 2014). The mammalian Par4 homologue liver kinase B1 (LKB1) activates AMP-activated protein kinase (AMPK), which phosphorylates CLIP-170, a +TIP, to enhance its association with the MT plus end and promote MT growth (Nakano and Takashima 2012). LKB1 also activates mammalian Par1b, which functions as a MT-associated protein kinase and phosphorylates MAP2, MAP4, and Tau to induce their dissociation from the lattice and increase dynamic instability (Ebneth, Drewes et al. 1999). RhoA signaling induces MT stabilization by signaling diaphanous-related formins (mDia) to interact with EB1, another +TIP, and APC, capturing the MT plus end and stabilizing the MT to allow for the accumulation of PTMs (Palazzo, Cook et al. 2001; Wen, Eng et al. 2004). Active Cdc42 interacts with Par6 to recruit the Par3/Par6/aPKC complex to the leading edge in migrating cells. aPKC downregulates GSK3β signaling, which allows APC to interact with and cap the MT plus end (Etienne-Manneville and Hall 2003). Par3 recruits dynein to

the cortex, which then pulls on these stabilized MTs and maintains centrosome positioning as actin flow moves the nucleus rearward, orienting the centrosome to the leading edge (Schmoranzer, Fawcett et al. 2009). Rac1 activation in migrating PtK1 epithelial promotes MT growth in cells by inactivating stathmin/Op18, a protein that sequesters tubulin dimers and inhibits MT polymerization (Wittmann, Bokoch et al. 2004). Rac1 also promotes CLASP-2 binding and stabilization of MTs (Lansbergen, Grigoriev et al. 2006). The actions of these proteins have been observed in many polarizing cell types, including neurons during growth cone development, T-cells during formation of the immunological synapse, and in epithelial cells undergoing epithelial-to-mesenchymal transitions, and in a variety of migrating cells (Li and Gundersen 2008).

MTs can also regulate Rho GTPase signaling by sequestering or localizing GEFs. Tctex-1, a dynein light chain, has been shown to interact with and inactivate Lfc, a RhoA GEF, which downregulates RhoA signaling and allows for Rac1 and Cdc42 activation (Krendel, Zenke et al. 2002). Par1b can phosphorylate GEF-H1, a RhoA GEF, to release it from MTs and promote RhoA activity (Yoshimura and Miki 2011). MAP1B, a MAP that accumulates in the growing axon, recruits Tiam1/2, a Rac GEF, and activates Rac1 and Cdc42 in the axonal growth cone (Montenegro-Venegas, Tortosa et al. 2010).

In epithelial cells, three main polarity complexes maintain apical-basolateral polarity: Par3 (Par3/Par6/aPKC), Crumbs (Crumbs/PatJ/Pals1), and Scribble (Scribble/Dlg/Lgl) (Rodriguez-Boulan and Macara 2014). The Crumbs and Scribble complexes are epithelial specific and were both discovered in *Drosophila* genetic screens to be required for epithelial tissue formation (Tepass, Theres et al. 1990; Bilder, Li et al. 2000). In vertebrate

epithelia, Par3 localizes to tight junctions and passes Par6/aPKC to the Crumbs complex in the apical membrane, while Scribble localizes to the lateral membrane beneath tight junctions. Par3 and Crumbs complexes are mutually antagonistic of Scribble, which keeps complexes active in their own domain and prevents diffusion into other areas (Bilder, Schober et al. 2003). LKB1 (Par4) and Par1b also have established roles in epithelial polarity. Overexpression of STRAD, a pseudokinase that interacts with and activates LKB1, is sufficient to induce apical-basal polarity in single, non-contacting intestinal cells (Baas, Kuipers et al. 2004). Par1 maintains a lateral localization through mutual antagonism with aPKC, and overexpression can induce columnar epithelia to reorient the vertical apical surface to the lateral surface, similar to bile canniculi observed in hepatic epithelia (Cohen, Brennwald et al. 2004).

MDCK cells as a model for epithelial polarity

Developing *D. melangoster*, *C. elegans*, and mouse epithelia have all been used to study epithelial tissue formation and function. In order to study polarization in more detail, cultured cell lines capable of polarization are required. Much of the work characterizing apical-basal polarization has been done in Madin Darby canine kidney (MDCK) cells, a non-transformed columnar epithelial cell line that forms an electrically tight, polarized monolayer when cultured on permeable filters (Cereijido, Robbins et al. 1978; Rodriguez-Boulan, Kreitzer et al. 2005). Cells orient their basal surface to contact the surface of the culture dish, or other support, their lateral surface at cell-cell contacts, and apical surface facing the culturing media. Cells cultured on a two-dimensional flat surface establish cell-substrate contacts as soon as they are plated, immediately receiving polarization cues. GP135, a heavily

glycosylated calyx protein, determines the location of the apical surface by localizing to the non-contacting, free surface of the cell as soon as cells attach to a flat substrate (Meder, Shevchenko et al. 2005). The glycosylated extracellular domain is hypothesized to have ‘anti-adhesive’ properties and prevent cell-cell contacts from forming while the cytosolic domain links to the cortical actin cytoskeleton through Na/H+ exchange regulatory cofactor (NHERF1) and Ezrin. To circumvent these strong polarity cues initiated by cell contact with solid supports, many studies are done using cells cultured in a 3D protein matrix made of basement membrane proteins (collagen, fibronectin, laminin, etc.), which is more biologically relevant to tissue development. This isotropic environment removes the initial directional contact cue and makes cells more responsive to alterations in the early polarization machinery (Martin-Belmonte and Mostov 2008). As cells divide in this 3D environment, they form cysts comprised of a single layer of cells surrounding an inward-facing, fluid filled, apical lumen and an ECM-facing basolateral surface.

Cell-cell junctions and the cytoskeleton

At MDCK cell-cell contacts, two junctional complexes interact with the actin and MT cytoskeleton and maintain polarity (Green, Getsios et al. 2010). Tight junctions are localized just beneath the apical surface and are composed of Occludins, Claudins, and Junctional Adhesion Molecules (JAMs), transmembrane proteins that form homophilic interactions between cells to create a paracellular barrier. Tight junction organization is induced and maintained by cytoplasmic zonula occludens proteins (ZOs), which interact with the cytoplasmic tail of Claudin (Umeda, Ikenouchi et al. 2006). Adherens junctions are localized at the lateral surface beneath tight junctions and are composed of transmembrane nectins and E-Cadherin, which also form

homophilic interactions between cells. Calcium-dependent E-Cadherin interactions signal the recruitment of cytoplasmic α-catenin, β-catenin, and p120 catenin, which interact with the cytoskeleton. Both junctions are vital for establishing apico-basal polarity as JAMs recruit and localize Par3 near the apical surface (Itoh, Sasaki et al. 2001) and cadherin-cadherin contacts are sufficient to induce basolateral protein segregation (Charnley, Kroschewski et al. 2012). Loss of cadherin-cadherin interaction induces breakdown of tight junctions and internalization of the apical surface into a large, vacuolar compartment termed the vacuolar apical compartment (VAC), since it contains all apical markers and excludes basolateral markers (Vega-Salas, Salas et al. 1987). The localization of these junctions differ in *D. melangoster*, where adherens junctions localize above septate (tight) junctions, and in *C. elegans*, which has one homologous junctional zone containing both tight and adherens junction components (Rodriguez-Boulan and Macara 2014).

Junction formation and function is tightly linked to the cytoskeleton. Adherens junctions recruit F-actin via α-catenin to form a peripheral actin belt beneath the apical surface, which in turn organizes adherens and tight junctions into a continuous belt (Takeichi 2014). Tight junction formation recruits myosin-2 to constrict the peripheral actin, which maintains epithelial shape by constricting the apical membrane (Yamazaki, Umeda et al. 2008). Both dynamic and stable MTs are required for adherens junction turnover and maintenance (Chen, Kojima et al. 2003; Ivanov, McCall et al. 2006; Stehbens, Paterson et al. 2006; Meng, Mushika et al. 2008). Adherens junctions also influence MT organization, as cadherin-cadherin contacts are sufficient to induce MT stabilization (Chausovsky, Bershadsky et al. 2000). β-catenin recruits dynein to potentially pull MTs for centrosome positioning, while p120

catenin can anchor non-centrosomal MT minus ends through NEZHA and ninein (Ligon, Karki et al. 2001; Meng, Mushika et al. 2008). MT depolymerization inhibits tight junction barrier function, which also inhibits the delivery of myosin-2 to peripheral actin in epidermal epithelial cells (Sumigray, Foote et al. 2012).

Rho family GTPases and their effectors are also major players in coordinating both cytoskeletal remodeling and adhesions during development, as rho mutants disrupt barrier formation and function (Popoff and Geny 2009). However, it is unknown whether rho signaling directly affects junction formation or if these are downstream effects from their roles as general polarity regulators. Many RhoA GEFs localize to adherens junctions to activate Rho activity, which in turn activates myosin-2 to induce constriction of the peripheral actin belt (Takeichi 2014). Rho-mediated acto-myosin constriction is coordinated between cells and is required for epithelial tissue remodeling, dorsal closure in developing *D. melangoster*, and closure of epithelial sheets during wound repair (Kiehart, Galbraith et al. 2000; Nakajima and Tanoue 2010; Antunes, Pereira et al. 2013).

Cell-substrate contact and integrin signaling

MDCK cysts grown in ECM have an inward-facing apical lumen and ECM-facing basolateral surface, while cysts grown suspended in media have an inverted polarity, orienting the apical membrane to face the media and secreting ECM proteins in the enclosed basolateral membrane (Wang, Ojakian et al. 1990). Embedding suspension-grown cysts in collagen will induce a reversal in polarity, reorienting the apical membrane to an inner lumen and basolateral membrane out towards the ECM (Wang, Ojakian et al. 1990). Additionally, overlaying collagen on the apical surface of polarized cells will

induce the apical membrane to internalize and relocalize to lateral lumens (Ojakian, Nelson et al. 1997), suggesting that cell-substrate contacts are sufficient to orient epithelial polarity. More detailed studies have revealed that the composition of the ECM can influence polarization. Single cells embedded in collagen through a process known as 'hollowing', where cysts form lumens after multiple rounds of cell division by clearing cells from the center (Martin-Belmonte, Yu et al. 2008). Cells embedded in Matrigel™, which contains a mixture of ECM proteins (collagen, laminin, fibronectin, etc.), polarize via 'cavitation', forming a lumen after one or two rounds of cell division by transcytosing the apical membrane into the lateral space. Later studies revealed that β1-integrin binds to and organizes extracellular laminin, forming a complex with α2/α3 integrin to signal the downregulation of RhoA and upregulation Rac1 activity (O'Brien, Jou et al. 2001; Yu, Datta et al. 2005).

Phospholipid composition of apical and basolateral membrane domains

Different phospholipids are enriched in specific membranes and distinguish distinct domains in polarizing cells. Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) marks the trailing edge of migrating cells and the apical surface of polarized epithelia, while phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3) marks the tip of the growing axon, the leading edge of migrating cells, and the basolateral surface of epithelia (Shewan, Eastburn et al. 2011). PTEN, which dephosphorylates PI(3,4,5)P3 to PI(4,5)P2, localizes to the apical membrane initiation site (AMIS) prior to apical lumen formation in MDCK cysts (Martin-Belmonte, Gassama et al. 2007), while PI3K, which phosphorylates PI(4,5)P2 to PI(3,4,5)P3, localizes to the basolateral membrane (Gassama-Diagne, Yu et al. 2006). Introducing exogenous PI(3,4,5)P3 to the apical membrane of polarized monolayers induces

membrane protrusion and is sufficient to localize basolateral proteins to the apical membrane, while PI(4,5)P2 insertion into the basolateral membrane of polarized cysts is sufficient to relocalize apical proteins to the basolateral membrane. These phospholipids can operate downstream of the major polarity complexes, as Par3 recruits PTEN and Dlg, part of the lateral Scribble complex, recruits PI3K, while they operate upstream of Rho GTPases, as PI(4,5)P2 recruits Annexin2 to recruit Cdc42 and PI(3,4,5)P3 recruits Rac1 (Rodriguez-Boulan and Macara 2014).

De novo apical lumen formation

Studies in 3D MDCK cysts have revealed many of the steps needed for cells to establish an apical membrane (Martin-Belmonte and Mostov 2008). Cells do not receive their first polarity cues until the first cell division, at which point GP135 is excluded from the newly formed cell-cell contacts and segregated to the ECM-facing surface. After this segregation, ECM-integrin interactions signal β1 integrin to complex with FAK and p190RhoGAP (Bryant, Roignot et al. 2014). This represses RhoA-ROCK1 activation to no longer inhibit PKCBII. PKCBII phosphorylates NHERF1, which then dissociates from GP135. This is believed to trigger GP135 endocytosis as its interaction with Ezrin is lost, unanchoring it from the cortical actin cytoskeleton. Endocytosed GP135 localizes to a Rab11A/Rab8 compartment with microvillar components and other apical membrane proteins, similar to the apical recycling endosome (Ferrari, Veligodskiy et al. 2008). This compartment is the equivalent of the VAC observed in cells cultured in low calcium on 2D supports, based on its size and because it excludes basolateral proteins. To distinguish the AMIS from the lateral membrane, PTEN is recruited through an unknown mechanism to the cell-cell contacting surface and converts PI(3,4,5)P3 to

PI(4,5)P2, which recruits Annexin-2 and Cdc42 (Martin-Belmonte, Gassama et al. 2007). This is believed to promote lumen formation by reorganizing cortical actin for exocytosis and establishes apical identity by recruiting Par3/Par6/aPKC. At this point, three sets of trafficking machineries are thought to regulate exocytosis at the AMIS. First, Rab11 recruits FIP5, a Rab11 binding protein, and sorting nexin 18 (SNX18), which may mediate compartment tubulation and scission of GP135 vesicles (Willenborg, Jing et al. 2011). Second, Rab11/Rab8/Rabin8 recruits Sec15A, part of the exocyst complex, Tuba, a Cdc42 GEF, and Myosin5B, which is believed to regulate targeted GP135 vesicle transport and exocytosis at the AMIS (Bryant, Datta et al. 2010; Roland, Bryant et al. 2011). Finally, Rab27 recruits Slp2a, a synaptotagmin-like protein that tethers vesicles to the PI(4,5)P2-enriched AMIS, while Rab3 recruits Slp4a to direct Syntaxin 3-mediated vesicle fusion (Galvez-Santisteban, Rodriguez-Fraticelli et al. 2012). This forms a pre-apical patch at the lateral surface between cells, which is expanded as water and ion channels pump fluid into the luminal space and more apical proteins are targeted to the lumen.

While this model is compelling, the data suggests that it is not complete and must be taken with a grain of salt. Nearly every protein was placed in this model because it localizes to the Rab11a compartment and causes a multiple-lumen phenotype in 3D cultured MDCK cells after multiple days when knocked down. This does not reveal the specific functions for these proteins during lumenogenesis since lumens are indeed forming, though probably due to compensation by other trafficking machineries, hence the reason for multiple lumens over singular. The assigned protein functions, while likely, is inferred by work in other systems. Live imaging of apical membrane transcytosis might

reveal more details about whether these proteins are required for vesicle fission, transport, targeting, or fusion. It is not likely that Myosin5B mediates vesicular transport from the Rab11 compartment to the AMIS as myosins are typically responsible for final transport steps through the actin-rich cell cortex (Bond, Brandstaetter et al. 2011). Short range myosin transport through the cortex to the AMIS is likely coupled with long range transport by kinesins to the cortex. Kinesin-myosin coupled transport has been well studied in melanocytes, where Kif3 mediates melanophore transport towards the cell periphery, handing it off to Myosin-5 for dispersal along actin cables (Wu, Bowers et al. 1998). Additionally, long range axonal transport by kinesins is coupled to myosin-mediated exocytosis at the synapse (Rudolf, Bittins et al. 2011). The rabs involved with lumen formation also indicate that kinesins may play a role in targeted vesicle transport, which will be discussed below.

Apical and basolateral sorting signals

In order to maintain polarity, proteins are sorted in the TGN and targeted to each surface using well characterized sorting signals (Cao, Surma et al. 2012). Basolateral targeting signals typically consist of an amino acid motif in the cytoplasmic domain of the protein. This signal is recognized by clathrin adaptors and proteins are transported to the basolateral surface in clathrin-coated vesicles (Deborde, Perret et al. 2008). Apical targeting signals, on the other hand, typically induce protein clustering and include O- and N-linked glycosylation, which are recognized and linked by intravesicular galectins, GPI-anchoring, which promotes clustering in lipid rafts, and oligomerization (Delacour, Koch et al. 2009; Weisz and Rodriguez-Boulan 2009). It has been hypothesized that by clustering together, these proteins induce budding of the membrane to create vesicles and concentrate apical

proteins while excluding basolateral proteins. However, it is still not known how cellular transport machineries recognizes these apical vesicles.

Microtubule organization in polarized epithelial cells

In polarized epithelia, MT organization was initially described as a mixed-polarity meshwork beneath the apical membrane and overlying the basal membrane, with bundled MTs longitudinally parallel to the lateral membrane and the majority of plus ends oriented basally (Bacallao, Antony et al. 1989). Orientation of these lateral bundles was determined by lysing the cell membrane and adding excess tubulin in high salt buffer, which induced formation of tubulin “hooks” extending perpendicularly from the MT lattice (McIntosh and Euteneuer 1984). These hooks curve clockwise or counterclockwise depending on which direction MT plus ends are oriented, and sectioning *en face* through the monolayer reveals orientation of longitudinal MTs. Subsequent observations have revealed additional subsets of MTs in these cells, including MTs with apically oriented plus-ends, as determined by tracking growing MT plus ends in living cells with a fluorescent +TIP (Jaulin, Xue et al. 2007), MTs with minus ends anchored at adherens junctions (Meng, Mushika et al. 2008), and modified MTs distributed beneath the apical pole (Quinones, Danowski et al. 2011). In mouse mammary epithelia cysts embedded in collagen, β1-integrin signals through intergrin-linked protein kinase (ILK) to interact with the +TIP EB1 and orient MT plus ends towards the basal surface (Akhtar and Streuli 2013). While this paper shows that EB1 interacts with ILK, this is most likely a transient interaction since prolonged ILK/EB1/MT-plus end interaction would result in stabilized MTs and allow for MT modification. Since modified MTs are distributed beneath the apical pole and not near the basal surface, a more likely

explanation would be that this interaction might direct EB1 associated MT plus-ends to grow along the basal surface to form the basal mesh.

It has been well established that MTs are required for maintaining apical fidelity in polarized epithelia. Treating cells with nocodazole, a MT depolymerizing drug, causes apical proteins to become non-polarized, localizing on both apical and basolateral membranes (Breitfeld, McKinnon et al. 1990). The defect in apical targeting when MTs are disrupted is likely due to selective mislocalization of apical t-SNAREs, which are needed for fusion of vesicles containing apical reporters selectively with the apical membrane (Kreitzer, Schmoranzer et al. 2003). However, basolateral t-SNAREs remain localized to the lateral membrane, which may be why basolateral proteins are not mistargeted. It is also speculated that apical mistargeting might also be due to postendocytic trafficking, since apical recycling is MT dependent while basolateral recycling is not (Musch 2004).

Studies have shown that some clonally-derived MDCK cells that do not reorganize MTs as described above maintain a perinuclear MT organization and are able to polarize separate apical and basal domains, suggesting that MT reorganization is not necessary to establish apical-basal polarity (Grindstaff, Bacallao et al. 1998). However, the results of this study are probably due to the fact that cells were cultured on a flat substrate, so they immediately receive basal cues through substrate contact apical cues as GP135 localizes to the free surface (Meder, Shevchenko et al. 2005). Since these clonal cell lines were not examined in an isotropic environment, such as a 3D protein matrix, the role of MTs during early polarization events in the absence of contacting cues has yet to be determined.

Kinesin motor proteins and epithelial polarization

Mammalian cells contain >40 known kinesin family motors that are taxonomically assigned to 14 different families (Hirokawa and Noda 2008). All kinesins are comprised of three primary domains, a head domain containing MT and ATP binding sites, a stalk containing coiled-coil motifs for oligomerization, and a tail that interacts with various adaptors and cargoes. Kinesin motor activity must be regulated to prevent futile ATP hydrolysis and MT track congestion (Verhey and Hammond 2009). Kinesins are regulated by many different mechanisms, including autoinhibition by head-tail interaction, activation following cargo recognition, and activation by phosphorylation.

In epithelial cells, the apical trafficking machinery remains poorly understood and few kinesin motors have been shown to transport proteins from the Golgi to the apical membrane. Kif5B, a kinesin-1 family member, is required for the transport of the apical marker p75, and KifC3, a minus-end directed kinesin-14 family member, is required for the transport of influenza HA (Noda, Okada et al. 2001; Jaulin, Xue et al. 2007). However, we still do not know how kinesins recognize apical cargoes, nor what role they may hold in establishing polarity. Multiple kinesins could be involved in targeted vesicle transport during lumen formation. Two kinesin-2 family members, Kif3A and Kif17, are required for apical lumen formation in MDCK cysts as revealed by a multiple lumen or no lumen phenotype following knockdown (Jaulin and Kreitzer 2010; Boehlke, Kotsis et al. 2013). Kif3A was shown to interact with FIP5 through its tail domain and is speculated to transport GP135/Rab11 endosomes to the AMIS on central spindle microtubules during telophase (Li, Kuehn et al. 2014). However, this interpretation may not be entirely correct, since the data from this thesis and other labs clearly show that lumen

formation occurs after cell division, once cell-cell junctions have been established. As such, it is more likely that Kif3A transports GP135 from the cell periphery after endocytosis to concentrate it near the AMIS, as both knockdown and dominant-negative mutant expression appear to disperse FIP5 vesicles. Additionally, Kif3A has been shown to mediate Par3 transport via APC to the growing axon in neurons, so it may be required to transport the Par3 complex to establish the AMIS before lumen formation (Shi, Cheng et al. 2004). For these reasons, it is likely that another kinesin mediates the final transport step to the AMIS. Kif17 is a likely candidate since it is also required for lumen formation in MDCK cysts and is known to perform coordinated intraflagellar transport (IFT) in the primary cilium with Kif3A (Scholey 2013). Kif17 also targets APC to MT plus ends in cell protrusions, and this localization of APC was correlated with the ability of epithelial cells to polarize (Mimori-Kiyosue, Matsui et al. 2007). Alternatively, since Rab27 and Rab3 are both required for vesicle tethering and fusion, it is possible that either may be used to regulate kinesin transport, as evidenced by examples in neurons. Kif1A/Kif1Bβ, kinesin-3 family members, transport synaptic vesicles by interacting with Rab3/DENN/MADD, while the kinesin-1 family transports the TrkB neurotrophin receptor in the axon via Rab27/Slp1 (Niwa, Tanaka et al. 2008; Arimura, Kimura et al. 2009).

Microtubule modifications and kinesins

Since MT stabilization accompanies polarization, it is likely that modified MTs are providing specific tracks for a subset of kinesins (Verhey and Gaertig 2007). This is especially likely for apical transport since modified MTs are enriched beneath the apical pole. Certain kinesins have been shown to preferentially bind to modified MTs. Kif5B has been shown to preferentially

interact with acetylated MTs in neurons to direct JIP1 to a single neurite prior to axon formation, and misdirects JIP1 to all neurites when MTs are saturated with acetylation (Reed, Cai et al. 2006). Kif5B is also known to preferentially bind detyrosinated MTs to position intermediate filaments in fibroblasts (Liao and Gundersen 1998; Kreitzer, Liao et al. 1999). Loss of MT polyglutamylation alters KIF1A and Kif1A cargo distribution in neurons, indicating that Kif1A preferentially interacts with polyglutamylated MTs (Ikegami, Heier et al. 2007).

Based on the current literature on epithelial cells and known mechanisms in other polarizing cell types, I hypothesize that MTs and kinesin motors play a role in apical membrane formation. However, whether MTs are even required during initial polarization steps in epithelial cells, or how the cytoskeleton changes in response to rho and par signals, is not well established. Many downstream events during polarization may be influenced by MTs, such as directing motors for targeted protein transport, influencing changes in cell architecture, or regulating polarization signals. While our previous work has shown that kinesins are required for lumen formation as well as directed transport of an apical protein, we do not know what signals direct them to the apical surface. This thesis will provide a foundation for answering these questions by characterizing MT reorganization during epithelial polarization to propose a role for MTs and MT motors in establishing an apical membrane.

CHAPTER TWO

DISTINCT SUBSETS OF MICROTUBULES REORGANIZE AROUND THE APICAL MEMBRANE DURING LUMEN FORMATION

Abstract

MTs are required for many processes during cell polarization, including axon specification in neurons, cell migration, and apical fidelity in polarized epithelial cells. How MTs influence epithelial cells during early polarization events is poorly understood. To investigate this, I used MT drugs to perturb the cytoskeleton and assessed whether MDCK cells were able to establish an apical membrane de novo using a 2D and 3D lumen formation assay. I then characterized how MTs reorganize during lumen formation by staining subsets of MTs and observing plus and minus end localization in fixed and live cells. Nocodazole-induced MT depolymerization and taxol-induced stabilization either delayed or halted lumen formation in both systems. MT staining revealed that two biochemically distinct subsets of MTs reorganize after the apical membrane endocytoses into the vacuolar apical compartment (VAC), and again following exocytosis into the lumen. An unmodified, dynamic subset of MTs organized radially around VACs and lumens, oriented towards the cell periphery, and a modified, stable subset of MTs organized circumferentially around VACs and against the lumen face. MT regrowth revealed that MTs are nucleated near the VAC/lumen and at the cell periphery, while live recordings of EB1-GFP revealed that growing MT plus ends are oriented both towards and away from the nascent apical membrane. These findings reveal a novel MT array that forms as the nascent apical membrane is transcytosed to the site of the lumen. Aspects of this array may be indicative of how MTs respond to polarization cues and how kinesins might recognize distinct tracks for

targeted transport. Since adherens junctions can induce microtubule stabilization and recruit dynein, it is likely that microtubules are stabilized and modified following cell-cell contact. Then dynein might provide a pulling force on stabilized MTs to reposition VACs prior to lumen formation. Finally, kinesins might use MT modifications to direct apical membrane exocytosis at the lateral membrane to form a lumen.

Introduction

MT reorganization is required for polarization in many cell types, as has been established by pharmacologically perturbing the cytoskeleton in these systems. In axonal growth cones, nocodozole-induced MT depolymerization inhibits responses to directional migration cues (Rajnicek, Foubister et al. 2006). Fission yeast fail to deliver polarizing factors to the cortex following MT depolymerization, which induces a random branching phenotype during division (Sawin and Snaith 2004). Taxol-induced MT stabilization inhibits fibroblast migration as dynamic MTs are required for focal adhesion turnover (Liao, Nagasaki et al. 1995). Other aspects of the MT array are also required for proper polarization. Centrosome repositioning is required for axon specification, cell migration, and immunological synapse formation (Li and Gundersen 2008). In MDCK cells, the centrosome must reposition between the nucleus and the apical membrane initiation site (AMIS) in order for the lumen to form (Rodriguez-Fraticelli, Auzan et al. 2012). MT modification is also hypothesized to be required for polarization. In neurons, one cellular process accumulates modified MTs prior to differentiating into the axon, and localized MT stabilization in a neuronal process before differentiation is sufficient to accumulate MT PTMs and distinguish a single axon, while stabilization in all neurites causes multiple axon formation (Witte, Neukirchen et al. 2008). While

MTs are required for apical fidelity in polarized epithelial cells (Breitfeld, McKinnon et al. 1990), whether MTs are required during epithelial polarization has yet to be addressed.

I investigated how perturbing MTs affects early epithelial polarization events and how the cytoskeleton reorganizes since many pathways and proteins required for polarization, such as par complexes, rho GTPases, and kinesins, are influenced by or directly influence MT reorganization and stability. To observe de novo apical membrane formation, MDCK cells were cultured in a 3D matrigel matrix. However, since post-experiment processing (fixing, staining, imaging) of cells grown in 3D culture can be difficult, I used a calcium switch on confluent MDCK monolayers to induce apical membrane internalization and lumen formation (Vega-Salas, Salas et al. 1988). This facilitated analysis of cytoskeletal arrays at high resolution, and data obtained using the 2D system were then compared with data from cells grown in 3D. This comparative analysis provides the experimental dexterity needed to visualize changes in MT arrays relative to the forming apical membrane while confirming results using a physiologically relevant system. Using these two systems, I characterized how MTs are organized in cells during lumen formation and determined how cytoskeletal perturbations affect early steps in epithelial polarization.

Results

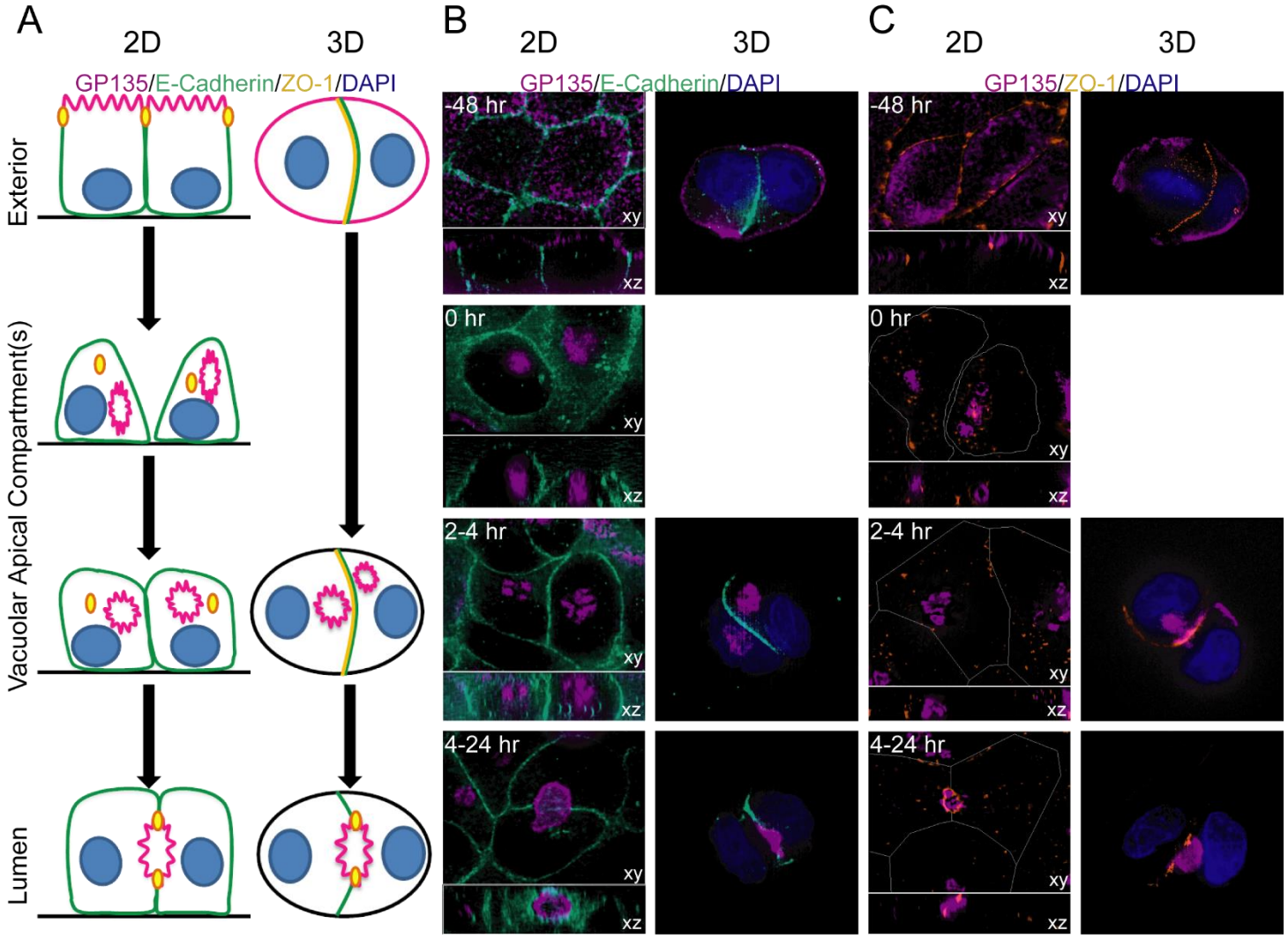
De novo apical membrane formation occurs similarly in 2d and 3d cultured cells

Two separate methods were utilized to observe early epithelial polarization events: a 2D calcium switch assay and a 3D cyst assay. Previous studies have shown that as epithelial cells begin to polarize in 2D, incubating

monolayers in a low calcium media induces apical membrane proteins to relocalize from the cell surface (exterior) to an internal vacuolar compartment (Figure 1A) (Vega-Salas, Salas et al. 1987). Low calcium treatment disengages the calcium-dependent cadherin-cadherin interactions between cells and disrupts cell-cell adhesions. Following a switch back to normal calcium levels, apical proteins localized to an interior lumen at the lateral surface. This mirrors what is observed in cells cultured in a 3D matrigel matrix, in which GP135 transcytoses to an interior lumen following cell division (Martin-Belmonte and Mostov 2008). In order to compare these two assays, I immunostained multiple markers for the apical membrane, adherens junctions, and tight junctions in fixed cells at discreet time points (Figure 1B, C). Prior to low calcium treatment in 2D cultured cells (-48 hours), GP135, an early apical marker, was localized at the exterior, “free” apical surface, separated from lateral E-Cadherin by tight junctions, as marked by ZO1. After cells were incubated in SMEM (5µm Ca^{2+}) for 48 hrs, GP135 relocalized from the exterior surface to internal vacuolar compartment(s) in 43.8% of cells (SEM=±2.4%, n=630) (0 hours). These compartments were VACs since they contained all tested apical markers (GP135, p75, GPI-anchored GFP) and actin, but did not contain basolateral markers (E-Cadherin, EGFR). Some E-Cadherin remained at the cell surface while internalized E-Cadherin and tight junction components (ZO1, Claudin-1) localized in intracellular puncta that were often near, but never colocalized with, apical proteins contained in VACs. These intracellular puncta were likely endosomes, as components of adherens and tight junctions are internalized by endocytosis when cell-cell adhesions are disrupted (Ivanov, Nusrat et al. 2005). Cells were then transferred to DMEM (1.8mM Ca^{2+}) to trigger re-assembly of cell-cell junctions, which reformed after 1-2 hours.

Figure 1: Lumen formation in 2D and 3D cultured cells

Comparisons between lumen formation in 2D and 3D are represented graphically (A) and via endogenous GP135 and E-Cadherin staining (B) or endogenous GP135 and ZO-1 staining (C). 2D cells are fixed at the indicated times during calcium switch and shown both en face (xy) and orthogonally (xz). 3D cysts are fixed between 24 and 33 hours after seeding.


A
2D
3D
GP135/E-Cadherin/ZO-1/DAPI
Exterior
Vacuolar Apical Compartment(s)
Lumen
B
2D
3D
GP135/E-Cadherin/DAPI
-48 hr
0 hr
2-4 hr
4-24 hr
xy
xz
C
2D
3D
GP135/ZO-1/DAPI
-48 hr
0 hr
2-4 hr
4-24 hr
xy
xz

At this time, VACs remained intact in the cytoplasm (2-4 hours). After 4 hours in DMEM, apical proteins localized at the lateral surface in 7.7% of cells (SEM=±2.5%, n=656), forming intercellular lumens (4-24 hours). Lumens were segregated from lateral membrane proteins by tight junctions. Between 4 and 8 hours after Ca^{2+} switch, the remaining apical proteins localized either to lumens or back to the exterior, free membrane. After 24 hours, apical proteins localized back to the exterior membrane in the majority of cells. Apical proteins followed a similar route when cells were cultured in 3D, with one key difference. Single MDCK cells seeded in matrigel formed cell-cell contacts after the first round of division (Figure 1B, 2). GP135 remained localized on the exterior cell surface in contact with the ECM while E-Cadherin and tight junctions localized to interior cell-cell contacts. Cells then internalized GP135 and other apical proteins into large rab11 positive structures, which were reminiscent of the VACs formed in 2D low Ca^{2+} culture and likely an apical recycling compartment, while cell-cell contacts remained intact. Note that this is where 3D culture differed from 2D, since 2D required cell-cell contact dissociation to form VACs while 3D required cell division and contact formation. GP135 then localized to an interior lumen between cells, separated from E-Cadherin by tight junctions. The similarities between 2D and 3D culture conditions indicates that the 2D system can be used to characterize MTs during lumen formation and compared to data obtained in 3D. To investigate MT organization and function, four different properties were determined: whether MTs are required for lumen formation, how MTs are organized during lumen formation, whether there are different MT subsets near the apical membrane, and how MTs are oriented near the apical membrane.

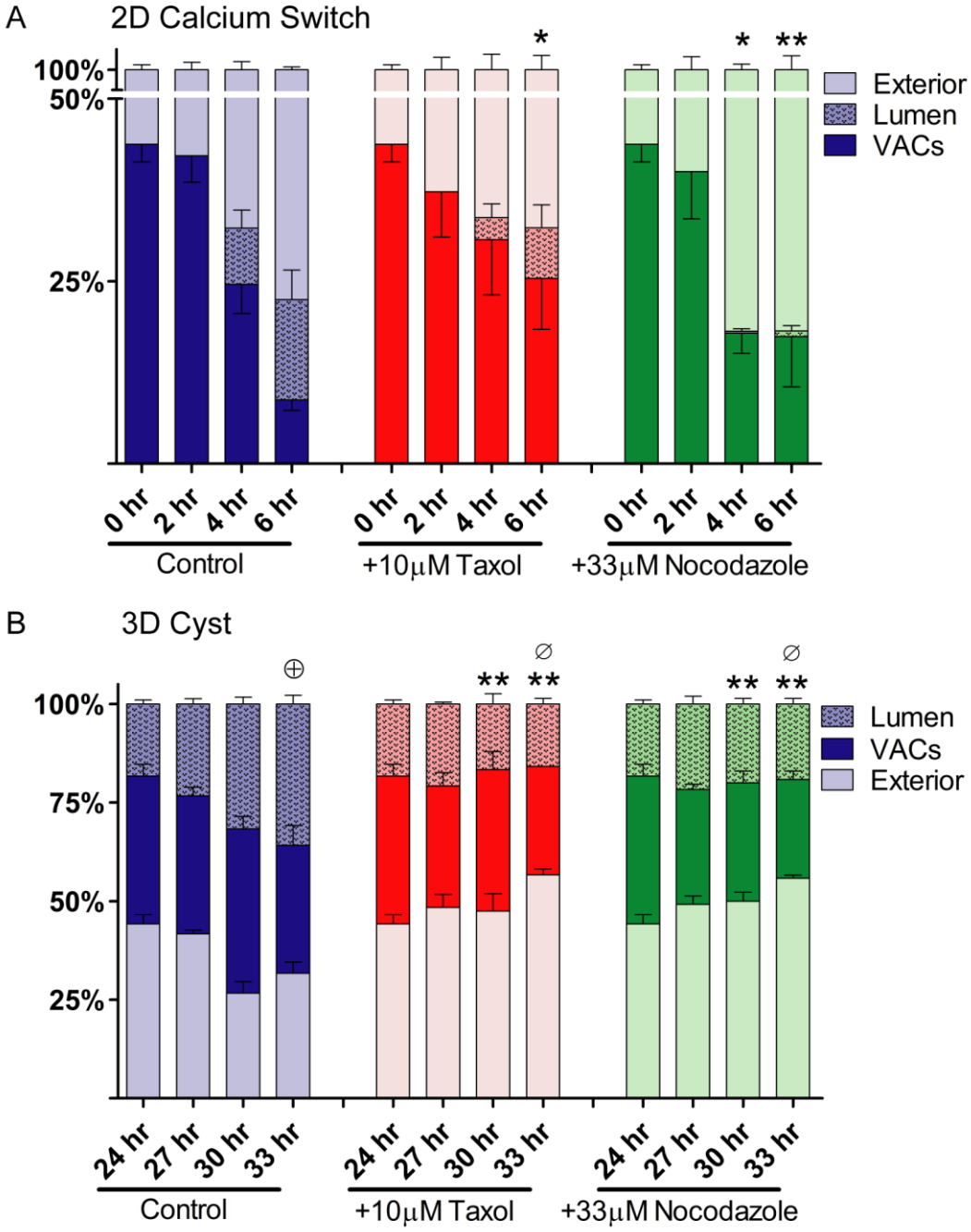
Microtubules are required for lumen formation

While it has been well established that MTs are required for apical fidelity in polarized epithelial cells, it is unknown whether MTs are required to establish an apical surface de novo during polarization. To address this question, I used a pharmacological approach to either depolymerize or stabilize MTs during lumen formation in both 2D and 3D systems and analyzed the effects of perturbation. In 2D cultured cells, I applied either 33µm nocodazole to depolymerize MTs or 10µm taxol to stabilize MTs just after calcium switch and fixed cells at 2 hour intervals. I scored cells based on whether GP135 localized to a VAC, lumen, or on the exterior membrane (Figure 2A). In control cells, GP135 localized to VACs in only 8.7% of cells 6 hours after calcium switch (SEM=±1.4%, n=608), while GP135 localized to lumens in 13.7% of cells (SEM=±4%). After 6 hours of taxol treatment, 25.3% of cells had GP135 localized in VACs (SEM=±6.9%, n=580), while 6.9% of cells had GP135 localized in lumens (SEM=±3%). Nocodazole treatment yielded a similar result after 6 hours (17.4% VACs; SEM=±6.9%, n=584), yet lumens appeared in <1% of cells (SEM=±0.7%).

To study the effects of MT perturbation on 3D cysts, single cells were seeded in matrigel and treated with 10µM taxol or 33µM nocodazole after 24 hours. Cells were fixed at 3 hour intervals between 24 and 33 hours post seeding and stained with GP135 and DAPI. Cysts containing two to four cells in interphase were scored based on GP135 localization, similarly to 2D (at the exterior membrane, to VACS, or to a lumen) (Figure 3B). During the 9 hour interval, control cysts shifted from the majority having GP135 localized on the exterior membrane (44% to 33%) to the majority having GP135 localized in an interior lumen (18% to 35%). However, both taxol and nocodazole treatment

Figure 2: Lumen formation over time following pharmacological perturbation of microtubules

Graphs represent localization of GP135 over time following 2D calcium switch (A) or during 3D cyst formation (B). Asters represent statistically significant differences between the number of cells or cysts with lumens as compared to control at the same time point. A) n>580 cells per time point, * $p<0.05$, ** $p<0.01$. B) n=120 cysts per time point, ** $p<0.01$. Alternate symbols indicate other statistical comparisons. In control cysts, the number of lumens at 33 hours is significantly more than at 24 hours (+, $p<0.01$). In drug treated cysts, the number of lumens at 33 hours is not significantly different than at 24 hours (Ø, $p>0.10$).


A
2D Calcium Switch
100%
50%
25%
Exterior
Lumen
VACs
*
*
**
0 hr
2 hr
4 hr
6 hr
Control
+10μM Taxol
+33μM Nocodazole
B
3D Cyst
100%
75%
50%
25%
Lumen
VACs
Exterior
⊕
∅
∅
**
**
**
**
24 hr
27 hr
30 hr
33 hr
Control
+10μM Taxol
+33μM Nocodazole

inhibited this shift, with the majority of cysts still having GP135 localized on the exterior membrane after 9 hours (56% and 55%, respectively).

The decrease in the number of cells with VACs coupled with the increase in cells with lumens indicates that GP135 exocytosed into the lateral membrane to form a lumen following calcium switch. However, since the number of VACs persisted longer in taxol treated cells, loss of dynamic MTs may have hindered exocytosis, though it did not completely inhibit lumen formation. Nocodazole treatment also did not inhibit exocytosis since the number of VACs decreased over time while more GP135 localized to the exterior membrane. However, it may have inhibit lumen formation due to mistargeting of other apical proteins to the basolateral membrane. In 3D experiments, the number of cysts with exterior-localized GP135 decreased while the number of cysts with lumen-localized GP135 increased significantly, indicating that GP135 transcytosed between 24 and 33 hours post-seeding. However, both taxol and nocodazole inhibited this shift, indicating that GP135 neither endocytosed into VACs, nor exocytosed to form a lumen. This indicates that MTs are required not only for maintaining apical fidelity, but also for establishing an apical membrane de novo. However, since this does not reveal the role MTs may play during lumen formation, I next investigated how MTs are organized to elucidate why MTs are required for lumen formation.

Microtubules reorganize during lumen formation

Since lumen formation either did not occur or was delayed when the MT cytoskeleton and its dynamics were disrupted, I examined MT organization during lumen formation to see if this could provide insight into how MTs affect this process. To visualize the MT cytoskeleton during lumen formation, cells were fixed during 2D calcium switch and 3D culture and stained for MTs and

actin. MT organization was noted specifically with respect to VACs or lumens, which were identified by bright actin staining. Prior to 2D calcium switch, MTs were organized as an apical mesh, basal mesh, and longitudinal bundles (Figure 3). Following 48 hours of low calcium treatment, MTs were arranged radially around VACs, extending to the cell periphery. MTs near VACs were either perpendicular to VACs, or circumferential around all sides of the VAC. In cases where there appeared to be multiple VACs clustered in a cell, circumferential MTs were more obvious, snaking between what were either individual structures or connected lobes of a larger structure. MTs did not reorganize after calcium switch and contact formation while VACs remained intact. After lumen formation, MTs reorganized to form a ‘radial center’ against the lumen face, extending to the opposite side of the cell. Some MTs perpendicular to the side of lumen appeared to enter or exit sites in the lumen where there were concave deformations. Additionally, a subset of MTs were organized parallel along the side of lumen, possibly similar to the circumferential MTs observed around VACs. MTs were organized similarly during lumen formation in 3D culture, where MTs organized radially around VACs or at the face of the lumen (Figure 3). MTs were either perpendicular to VACs and lumens, or circumferential along the sides of the membrane. The organizational changes observed during lumen formation reveal that MTs are reorganized during early epithelial polarization events. The two distinct orientations of MTs around VACs and lumens (perpendicular vs. circumferential) indicate that there may be differences between these MTs.

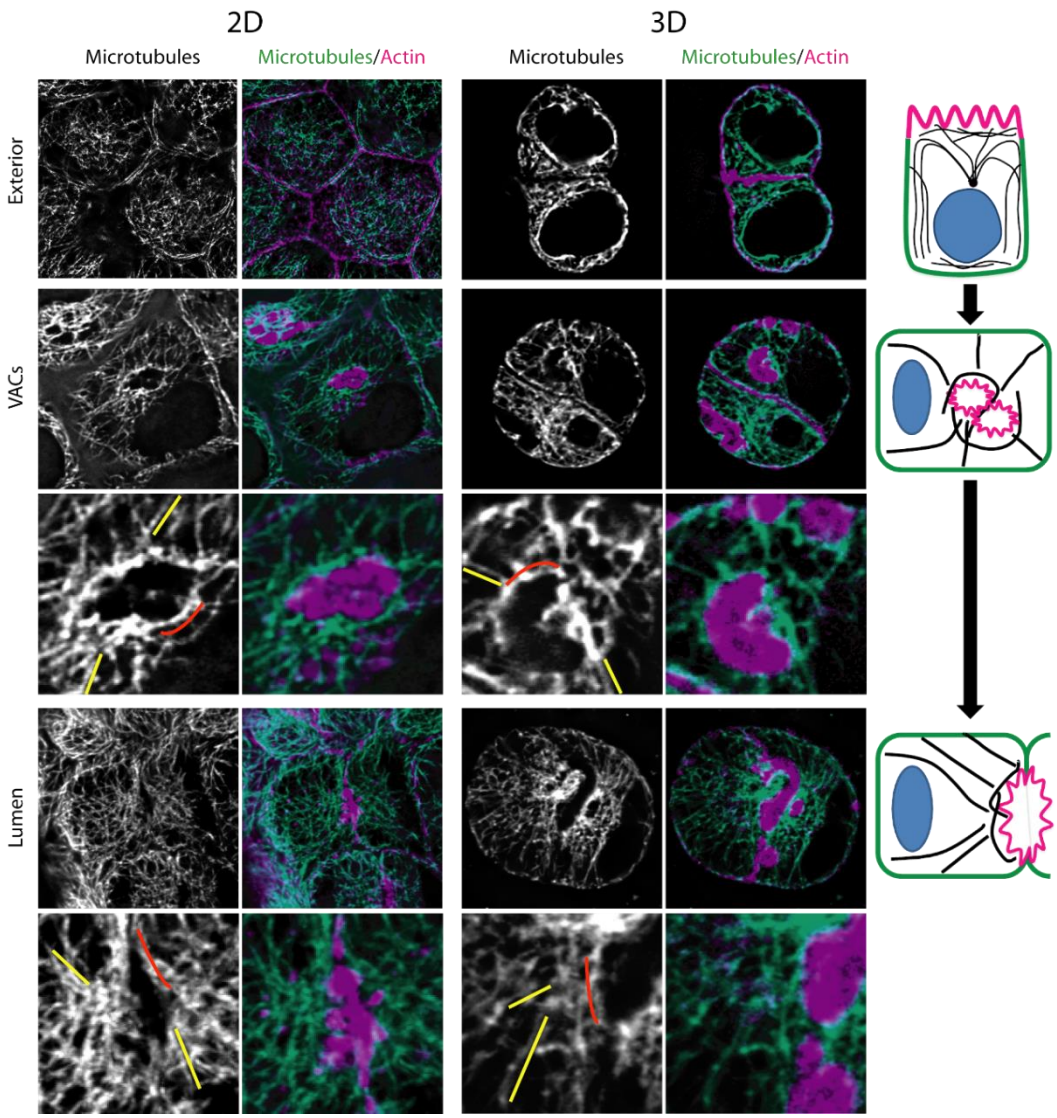


Figure 3: Microtubule organization during lumen formation in 2D and 3D

Representative images show MT organization near the apical surface with and without actin overlay. Large actin compartments show apical membrane localization, as labeled on the left. Panels below are enlargements of the area near the apical membrane. Colored lines are offset from MTs to highlight perpendicular (yellow line) versus circumferential (red curve) MTs. MT organization is represented graphically on the right. The first graphic represents a polarized cell (2D exterior) and not a nonpolarized cell (3D exterior).

Modified microtubules localize near the apical membrane during lumen formation

During cell polarization, MTs are stabilized in response to local cues. I noted that a subset of MTs did not depolymerize following cold and nocodazole treatment and were organized circumferentially around VACs (Figure 4). Nocodazole-resistant, stable MTs are marked by post-translational modifications (PTMs), since modifying enzymes preferentially interact with the assembled MT lattice while demodifying enzymes preferentially interact with soluble tubulin dimers (Song and Brady 2015). While the function of these modifications is still not well defined, certain kinesins and MAPs are known to preferentially bind to modified MTs, indicating that they may play a role in organelle positioning and directed membrane trafficking (Verhey and Gaertig 2007). To observe the distribution of stable, modified MTs during lumen formation, cells were stained with antibodies recognizing two well-known PTMs: acetylation (acetylated MTs), which is added to lysine 40 on the luminal side of α-tubulin, and detyrosination (glu MTs), in which the C-terminal tyrosine is removed from α-tubulin, leaving the penultimate glutamic acid (Figure 5, 6). A subset of MTs that still have N-terminal tyrosine (Tyr MTs) was also stained to distinguish the population of dynamic MTs. Prior to calcium switch, modified MTs were enriched beneath the apical pole of polarized cells (Figure 5). Following low calcium treatment in 2D, the majority of modified MTs localized near VACs and were distinctly circumferential, whereas unmodified MTs were mostly perpendicular to VACs and distributed throughout the entire cell. While most modified MTs were both acetylated and detyrosinated, a few regions on MTs were marked separately with only one modification (Figure 6). After calcium switch and lumen formation, modified MTs localized parallel

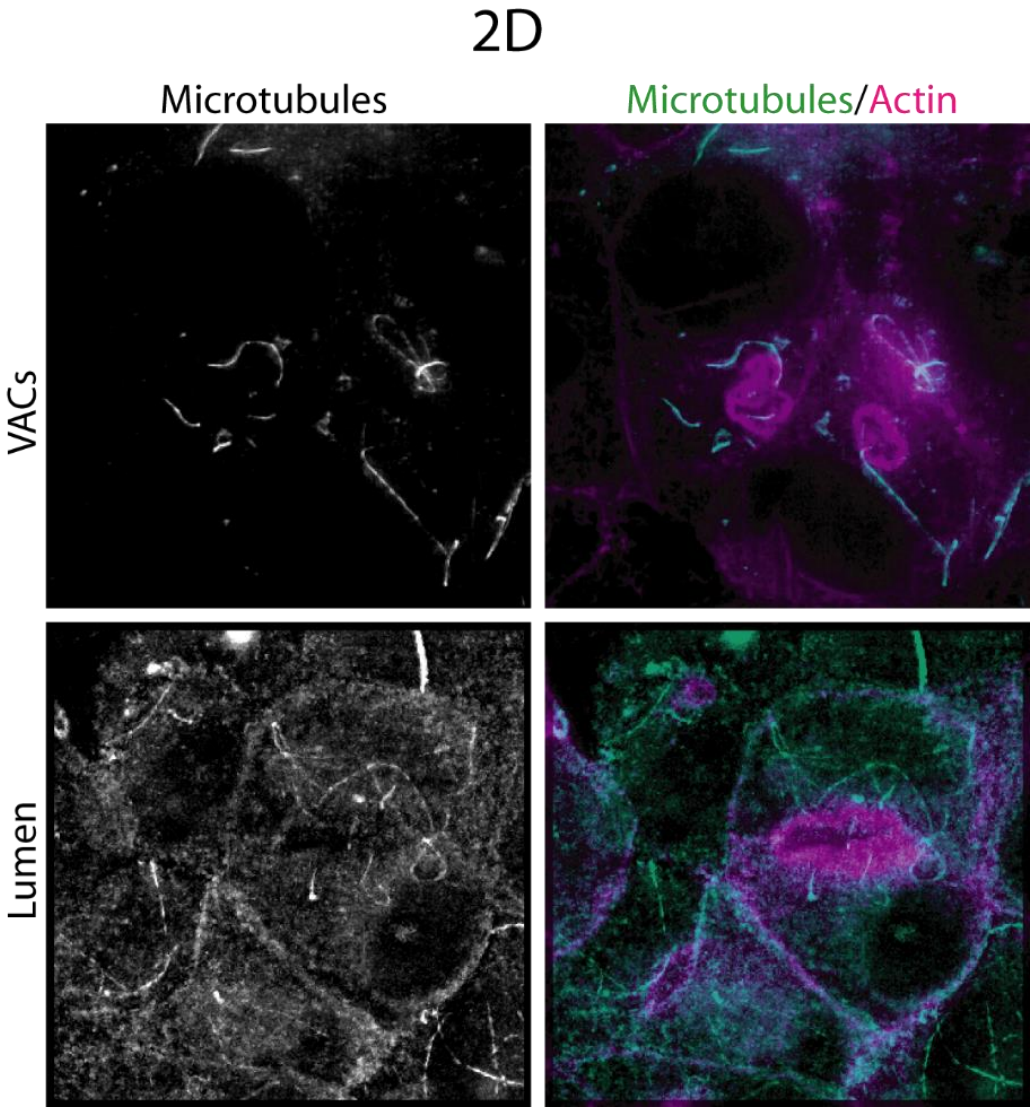


Figure 4: Cold- and nocodazole-resistant microtubule distribution during lumen formation in 2D

Representative images show MTs near the apical membrane following cold and nocodazole treatment after calcium switch. Actin overlay shows localization of the apical membrane, as labeled on the left.

Figure 5: Modified microtubule distribution during lumen formation in 2D and 3D

Representative images show modified (acetylated) and unmodified (tyr) MT distribution near the apical membrane. Actin overlay shows localization of the apical membrane, labeled on the left. 2D calcium switch is represented both en face (xy) and orthogonally (xz). Panels below are enlargements of the area near the apical membrane. Colored lines are offset from MTs to highlight perpendicular (yellow line) versus circumferential (red curve) MTs. Distribution of modified MTs is represented graphically on the right. The first graphic represents a polarized cell (2D exterior) and not a nonpolarized cell (3D exterior).

3D
Acet MTs/Actin
Acetylated MTs
2D
Acet MTs/Actin
xy
xz
Tyrosinated MTs
Acetylated MTs
Exterior
VACs
Lumen

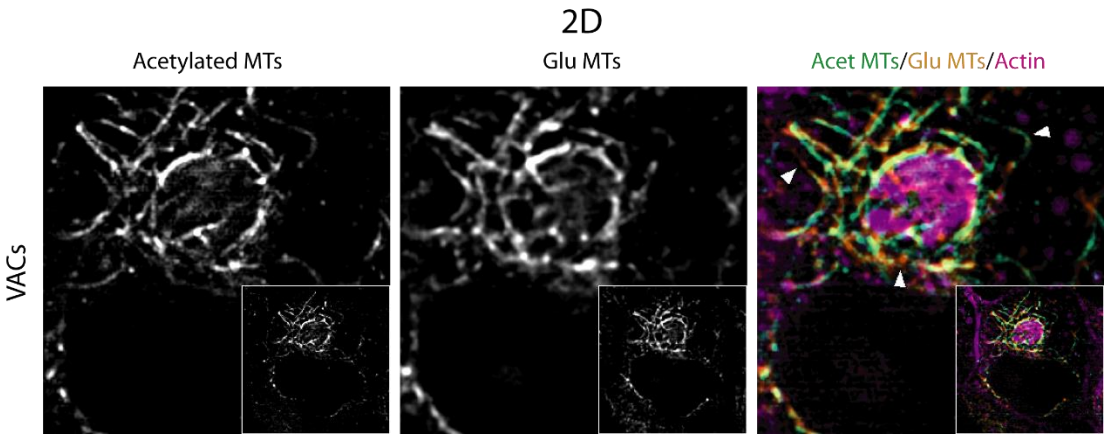


Figure 6: Acetylated and detyrosinated microtubule distribution around VACs

Representative image shows acetylated and glu MTs near the apical membrane. Actin overlay shows localization of the apical membrane in a VAC. Inset panels show the entire cell. Arrows point to modified MTs that are only acetylated or glu.

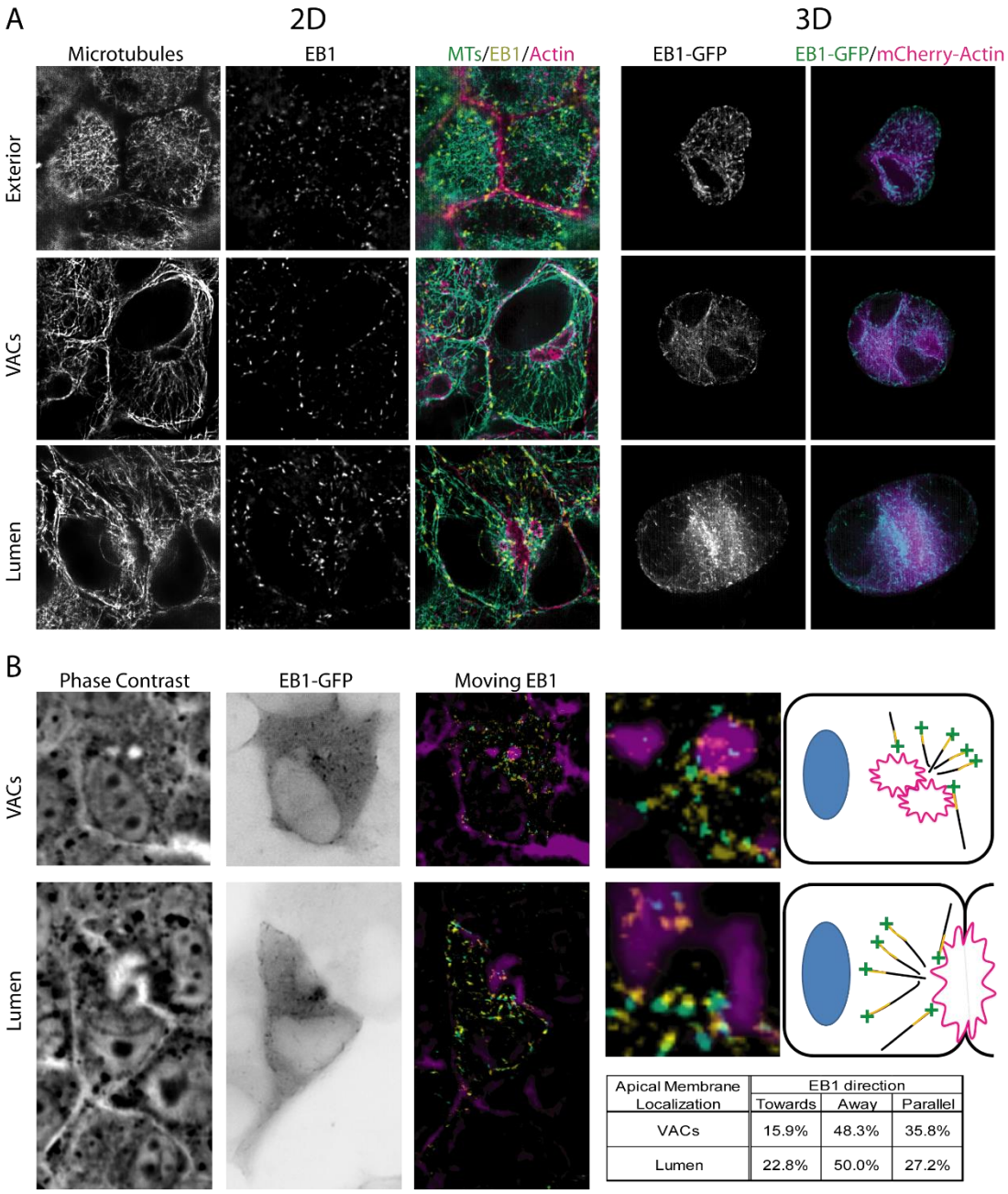
against the side of the lumen while unmodified MTs were localized throughout the entire cell. Modified MTs were often enriched in one of the two cells forming a lumen. In 3D cysts, modified MTs had a similar distribution around VACs and close to the lumen, though often only short sections of MTs were modified as opposed to the longer stretches observed in 2D cells (Figure 5). Since the organization of modified MTs around VACs changed following lumen formation, this suggests a link between lumen formation and MT stabilization.

Centrosomal and noncentrosomal microtubules are oriented bidirectionally around the apical membrane

Since MTs are required for lumen formation, they may provide tracks for membrane transport from the VAC to the lateral membrane. Two plus-end directed kinesins, Kif3 and Kif17, are required for lumen formation in 3D cultures (Jaulin and Kreitzer 2010; Boehlke, Kotsis et al. 2013), which suggests that if kinesin-mediated membrane transport is needed for lumen formation, then MT plus ends would orient toward the nascent apical membrane to facilitate targeted membrane trafficking. In order to determine where MT plus-ends are localized, I examined the distribution of EB1, a well-known plus-end interacting protein (+TIP) that tracks the tips of MTs and promotes growth. Prior to 2D low calcium treatment, endogenous EB1 localized to comets on MTs throughout the cell and beneath the apical surface (Figure 7A). Following low calcium treatment and calcium switch, EB1 puncta localized on radial MTs throughout the cell, while a number of comets localized directly next to VACs and lumens. To test whether these MT plus ends were growing dynamically toward or away from VACs and lumens, MDCK cells were transfected with EB1-GFP and imaged live to observe EB1

Figure 7: EB1 localization during lumen formation in 2D and 3D

A) Representative images show endogenous EB1 localization on microtubules in 2D and EB1-GFP localization in 3D. Actin overlay shows localization of the apical membrane, labeled on the left. B) Images show EB1-GFP in live cells. Phase contrast shows apical membrane localization at phase light spots. Grayscale image shows EB1-GFP from one frame. Color overlay shows subsequent frames overlayed and pseudocolored to show EB1 displacement (yellow) and directionality (final frame, cyan) over 10 seconds near the apical membrane (magenta). MT plus end localization is represented graphically on the right. C) Table represents direction of EB1 movement near the apical membrane. n=93 events in 13 cells (VACS) or n=115 events in 22 cells (lumens).



Apical Membrane Localization	EB1 direction		
	Towards	Away	Parallel
VACs	15.9%	48.3%	35.8%
Lumen	22.8%	50.0%	27.2%

tip tracking near the apical membrane (Figure 7B). EB1 comets emerged in aster-like patterns from sites adjacent to VACs or lumens, suggesting that these were sites of EB1 loading onto newly growing MTs near the centrosome, which was later confirmed by staining centrosomal markers (Figure 9). Moving EB1 comets near VACs or lumens were scored based on directionality if they moved persistently in the plane of view for at least 10 seconds. While nearly half of EB1 comets moved away from VACs or lumens, at least 15% of EB1 comets moved towards VACs or lumens, with the remaining 35% moving parallel to VACs or lumens. The distribution of EB1-GFP in fixed 3D cysts was similar, localizing throughout the cell and directly next to VACs and lumens (Figure 7A).

If plus-end directed transport is required for lumen formation, then MT minus ends would also need to localize near VACs to establish a route to the site of lumen formation. Previous studies indicate that the position of the centrosome is vital for lumen formation in MDCK cells (Rodriguez-Fraticelli, Auzan et al. 2012). Additionally, some minus-end directed motors have been shown to transport apical cargoes (Noda, Okada et al. 2001), indicating that minus ends need to be near VACs for their transport. To determine MT minus end localization, I stained MTs and actin following MTs regrowth in both 2D and 3D cultured cells (Figure 8A). MTs grew in asters adjacent to VACs or lumens, indicating that the centrosome was near the apical membrane. Other MTs regrew from three additional sites: at the cell periphery, in the cytoplasm, and at the apical membrane. Staining centrosomal and Golgi markers revealed that sites of MT nucleation were near VACs or the lumen in both 2D and 3D systems (Figure 9). Additionally, proteins known to anchor non-centrosomal MTs at the cell periphery, NEZHA and ninein, colocalized with

Figure 8: Microtubule growback following nocodazole washout during lumen formation in 2D and 3D

Representative images show MT growth 10 minutes after nocodazole washout. Actin overlay shows localization of the apical membrane, labeled on the left. Inset shows enlargement of MT regrowth from asters.

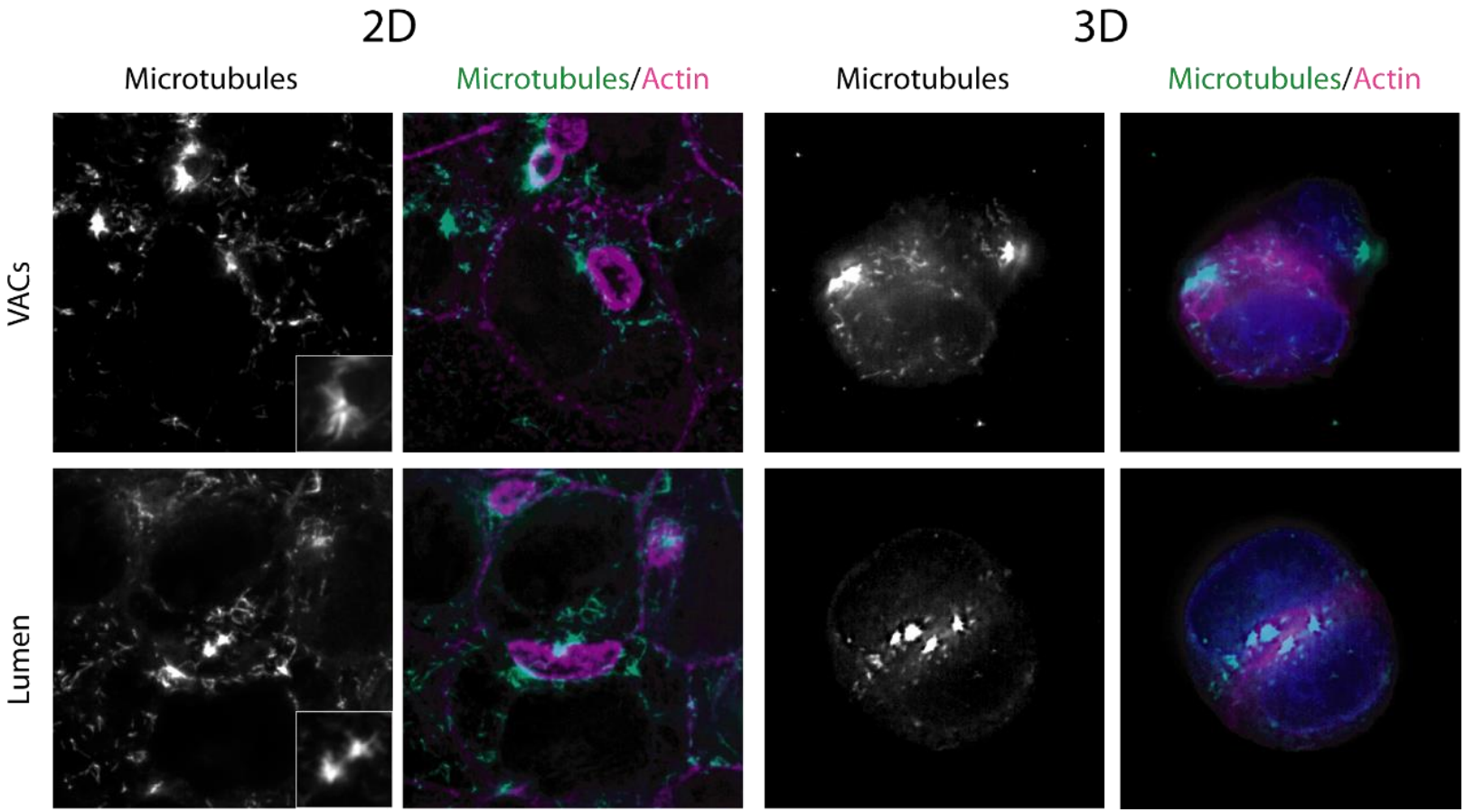
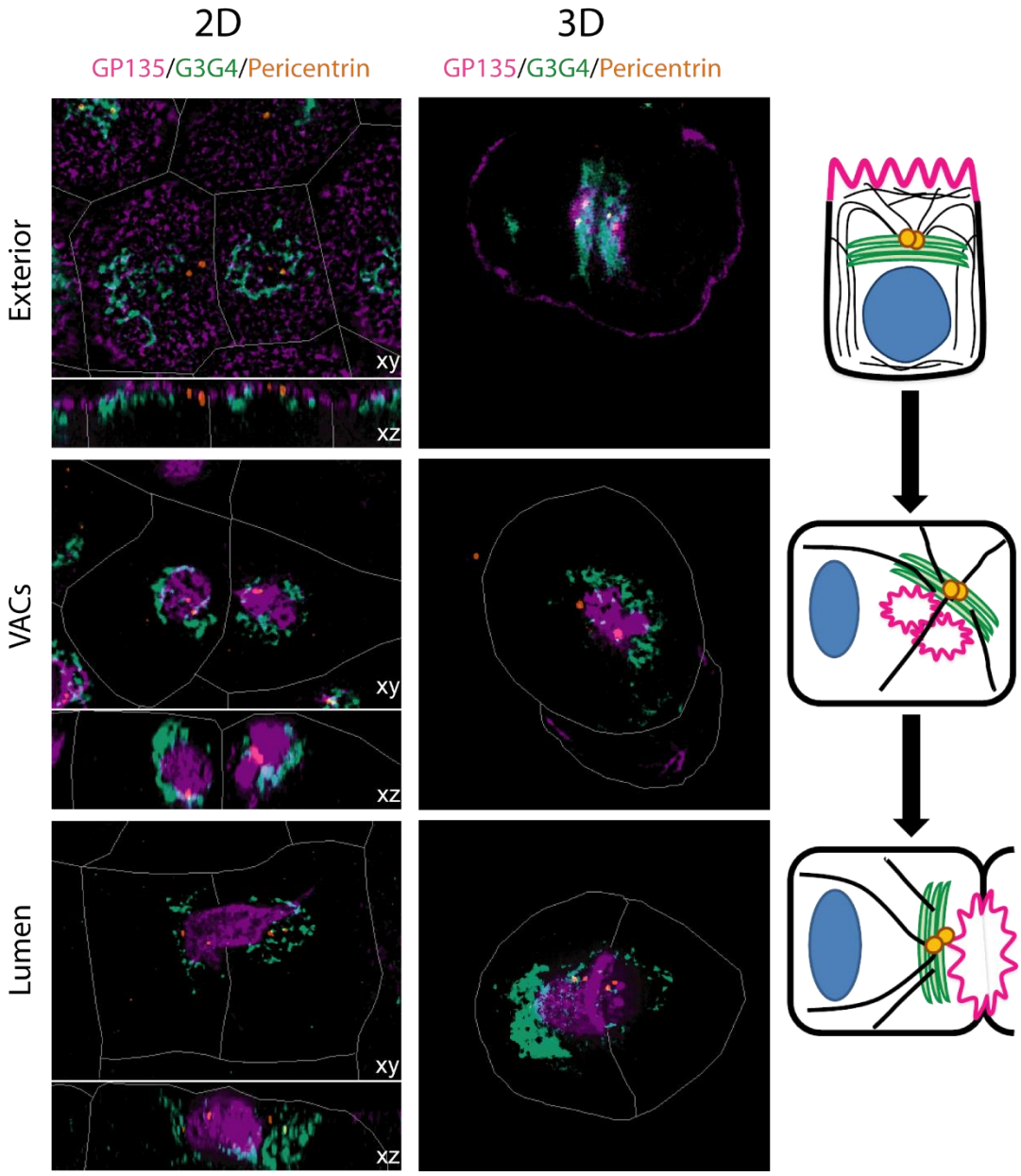

2D
3D
Microtubules
Microtubules/Actin
Microtubules
Microtubules/Actin
VACs
Lumen

Figure 9: Centrosome and Golgi localization during lumen formation in 2D and 3D

Representative images show pericentrin (centrosomal marker, orange) and G3G4 (Golgi marker, cyan) localization during lumen formation. GP135 (magenta) shows localization of the apical membrane, labeled on the left. 2D calcium switch is represented both en face (xy) and orthogonally (xz). Centrosome and Golgi localization during lumen formation is represented graphically on the right. The first graphic represents a polarized cell (2D exterior) and not a nonpolarized cell (3D exterior).


2D
3D
GP135/G3G4/Pericentrin
GP135/G3G4/Pericentrin
Exterior
VACs
Lumen
xy
xz
xy
xz
xy
xz

one end of some MTs (not shown) (Mogensen, Malik et al. 2000; Meng, Mushika et al. 2008). This suggests that MT minus ends localize in two places during lumen formation: adjacent to the VAC or lumen and at the cell periphery, and that they grow bidirectionally, either towards or away from the nascent apical surface. Therefore, both plus and minus-end directed transport can be used to maintain VACs and lumens.

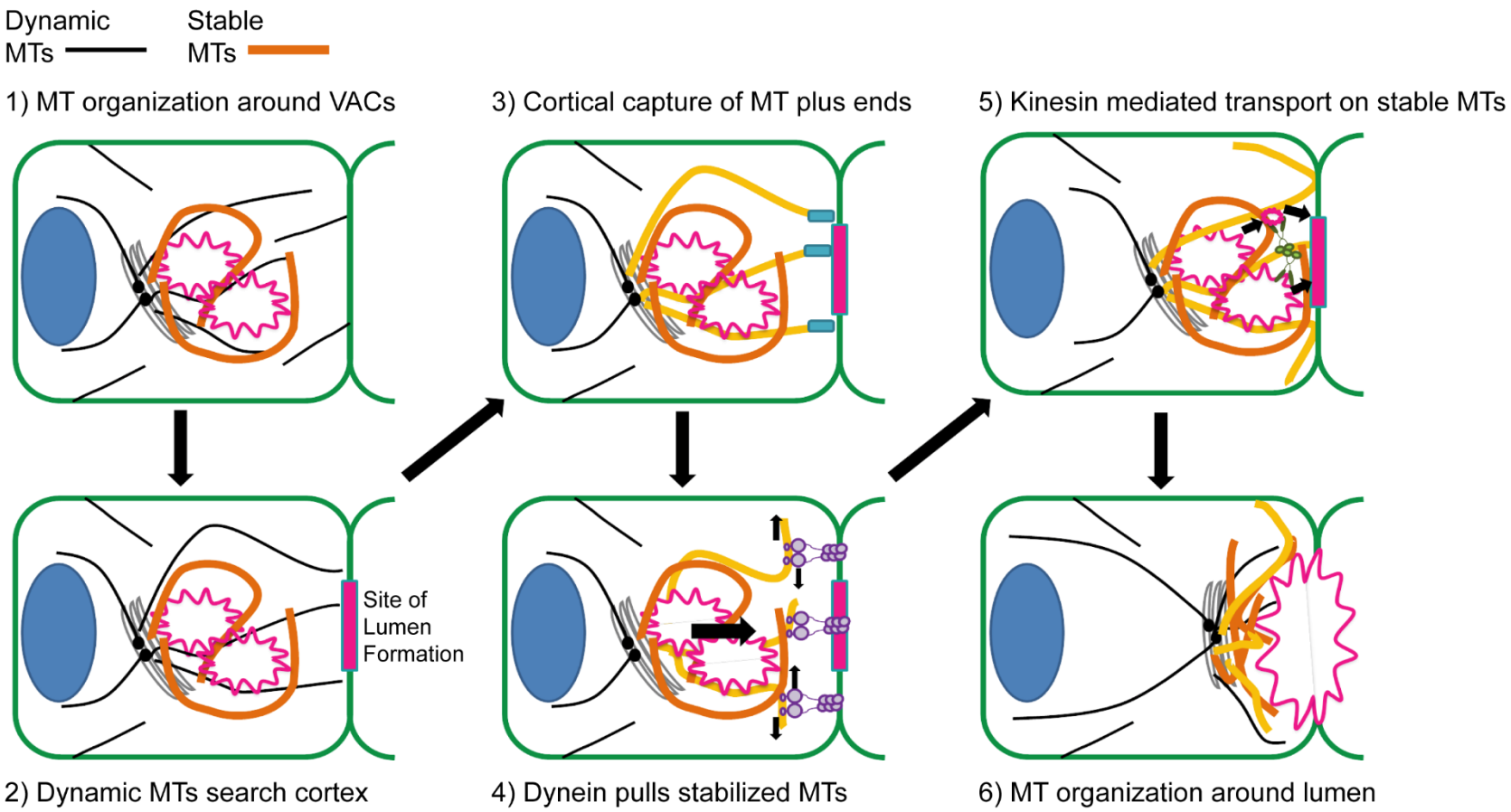
Discussion

This work describes, for the first time, how the MT cytoskeleton reorganizes during lumen formation in epithelial cells and adds further support for the hypothesis that MT reorganization is key in directing specific membrane trafficking events. Additionally, while it is well known that MTs are required for specifically targeting proteins to an established apical surface, I have shown that MTs are required for de novo formation of an apical surface. From this study, three subsets of MTs can be distinguished during lumen formation: dynamic MTs nucleated near the apical membrane, dynamic MTs anchored at the cell periphery, and stable MTs localized near the apical membrane (Figure 10).

The positioning and orientation of MTs around the VAC suggests potential mechanisms for how MTs contribute to lumen formation. Since modified MTs are localized next to the lumen, kinesins that preferentially associate with modified MTs may utilize them as tracks to transport apical proteins to the site of the lumen. I attempted to determine whether MT modifications influence lumen formation by treating cells with tubacin, which inhibits HDAC6 activity and prevents tubulin deacetylation, saturating MTs with acetylation. I scored cells similarly to those treated with nocodazole or taxol and found that tubacin treatment did not alter lumen formation in either 2D or

Figure 10: Microtubule reorganization during de novo apical membrane formation

Graphical representation shows distribution of dynamic and stable MTs with respect to the apical membrane (magenta), centrosome (black), and Golgi (gray) during lumen formation. Sequential images (1-6) indicate a hypothetical mechanism for MT reorganization, as will be discussed in chapter four.


Dynamic MTs
Stable MTs
1) MT organization around VACs
2) Dynamic MTs search cortex
Site of Lumen Formation
3) Cortical capture of MT plus ends
4) Dynein pulls stabilized MTs
5) Kinesin mediated transport on stable MTs
6) MT organization around lumen

3D. This does not necessarily indicate that modified MTs are dispensable for lumen formation. While the expected outcome of saturating MTs with modifications would be multiple lumen formation if the kinesins recognizing modified MTs are misdirected, lumen formation should only occur at the AMIS, where PI(4,5)P2 is localized to direct Slp2a/Slp4a-mediated vesicle tethering and other apical signaling events (Martin-Belmonte, Gassama et al. 2007; Galvez-Santisteban, Rodriguez-Fraticelli et al. 2012). Hindering MT modification would provide a better readout for whether modified MTs contribute to lumen formation. This could be done by knocking down αTAT1, which acetylates MTs, or by microinjecting cells with modified MT-specific antibodies to hinder kinesin-MT interactions. One study has suggested that overexpression of tubulin tyrosine ligase (TTL), which 'demodifies' detyrosinated tubulin dimers by adding back the C-terminal tyrosine to α tubulin, inhibits MT detyrosination and promotes faster polarization of 2D monolayers (Zink, Grosse et al. 2012). However, since TTL does not interact with the assembled MT lattice and would not be able to demodify long-lived MTs, the absence of detyrosinated MTs and resulting 'fast-polarization' defect might be an artifact from isolating a single clone that overexpressed TTL from a more homogenous population. Since the carboxypeptidase that mediates MT detyrosination is unknown, pharmacological manipulation is currently the only means of depleting cells of glu MTs. Okadaic acid, a protein phosphatase 1 and 2A inhibitor, has been shown to selectively induce breakdown of glu MTs (Gurland and Gundersen 1993), but may induce wide off-target effects. Alternatively, parthenolide, an NFκB signaling inhibitor, has been shown to inhibit MT detyrosination in HeLa cells (Fonrose, Ausseil et al. 2007), though I was unable to recapitulate these results in MDCK cells.

Since nocodazole-induced MT depolymerization halted apical membrane transcytosis and lumen formation in 3D cysts, it is highly likely that MTs either provide tracks for GP135 transcytosis, are needed to position where the VAC will form, or specify where lumens will form. However, nocodazole treatment during calcium switch likely inhibited lumen formation by causing apical proteins other than GP135 to mistarget to the basolateral membrane. Alternatively, nocodazole induces global RhoA activation (Chang, Nalbant et al. 2008), which has also been shown to inhibit polarization and may do so independently of the absence of MTs (Yu, Shewan et al. 2008). Taxol-mediated MT stabilization also arrested apical membrane transcytosis in 3D cysts, indicating that dynamic MTs are required for both GP135 endocytosis and lumen formation. Dynamic MTs continuously rearrange and probe the cell cortex, which could contribute to a variety of trafficking events. Prior to endocytosis, MTs may deliver trafficking machinery required for endocytic and transport events. Additionally, MTs may find sites at the ECM-facing surface to generate non-centrosomal MTs and provide tracks into the cell for endocytosis. Finally, MTs may deliver a GEF to affect Rac1 signaling, which is vital for initiating laminin organization and signaling GP135 endocytosis following β1 integrin activation (Yu, Datta et al. 2005). Following endocytosis and leading up to lumen formation, dynamic MTs may probe the cortex at cell contacts to encounter stabilization signals, as indicated by the modified, stable MT distribution near the lumen. This model will be discussed in chapter four.

Using the 2D calcium switch, new questions can be posed as to how the apical surface is trafficked during lumen formation. While it is known that the apical surface localizes from the exterior membrane to an internalized VAC

to an interior lumen in live 3D cysts (Ferrari, Veligodskiy et al. 2008), it has not been definitively shown that VACs form from endocytosed exterior membrane and may instead be formed by de novo protein synthesis and trafficking through the secretory pathway. Additionally, it has not been shown whether the lumen is formed by en masse exocytosis of the VAC, vesiculation and transport of VACs, or from de novo protein synthesis following VAC degradation. My initial studies observing surface-labeled p75 localization in 2D cultured cells suggests that the apical surface is endocytosed into VACs and re-exocytosed into lumens following calcium switch since surface-labeled p75 was present in the lumen. Live imaging also suggests that VACs are vesiculated and targeted to the site of the lumen, since VACs shrank over time as lumens appeared. However, en masse exocytosis may still occur since certain lumens in fixed cells appear connected by a thin tubule of membrane to an internal round membrane, reminiscent of a VAC. Finally, how and why the apical surface relocalizes from the lumen to the exterior membrane in 2D calcium switch is unknown. ZO-1 and E-Cadherin staining reveals that the lumen is surrounded on all sides by adhesions, so the apical membrane either transcytoses to the free surface or junctions open to allow proteins and lipids to diffuse back to the top of the cell. Many of these questions can be answered by live imaging calcium switched cells or 3D cysts and following a fluorescently tagged apical marker from the exterior membrane to the lumen.

Although they appear similar, I cannot resolve whether the apical membrane is trafficked via the same pathway in both 2D and 3D systems since the signals triggering apical membrane endocytosis are different. In 3D cysts, integrin-ECM interactions downregulate RhoA to upregulate Rac1 and signal apical membrane transcytosis (Yu, Shewan et al. 2008). Loss of

cadherin-cadherin interactions following 2D calcium switch may downregulate RhoA as well, since RhoA function is linked with junctions and peripheral actin. RhoA and Rac1 are mutually antagonistic, so this potential loss of RhoA signaling may stimulate Rac1 and allow for PKCBII activation, which induces GP135 endocytosis in 3D (Bryant, Roignot et al. 2014). To test whether VACs are formed similarly in 2D and 3D, knockdown or inhibition of these factors could reveal whether they mediate the same events in 2D if VACs no longer form in low calcium. However, since only ~40% of cells form internal VACs and nearly all cells lose cell-cell contacts, other unknown factors may be at play to induce apical membrane endocytosis in 2D.

MTs may also be responsible for VAC and lumen positioning and maintenance. MTs and MT motors are required for maintaining the structure and positioning of many membranous organelles, such as the ER, Golgi, mitochondria, and nucleus (de Forges, Bouissou et al. 2012). Since MTs are oriented bidirectionally around VACs, both plus and minus-end directed motors can be targeted to VACs. This is important for VAC maintenance, since the few trafficking mechanisms identified for apical proteins use plus or minus-end directed motors (Weisz and Rodriguez-Boulan 2009). MTs and EB1 comets were observed concentrated in concave deformations in VACs and lumens, which indicates that MT plus ends may provide a pushing force on the apical membrane. However, since extended periods of nocodazole-induced depolymerization did not cause a notable disruption of VAC size or positioning, stable, nocodazole-resistant MTs may provide positioning cues for VACs. Additionally, I've observed KIF5B localized to the VAC surface, which is known to associate with modified MTs and has roles in organelle positioning and apical trafficking. To test which MT motors might maintain VAC positioning and

size, motors known to either traffic apical cargoes or position organelles could be inhibited or knocked down and the size and distribution of VACs compared to control cells.

Materials and methods

Cell culture and transfection

MDCK II (Philadelphia clone) were cultured in DMEM (4.5 g/L glucose) supplemented with 5% FBS and 20mM Hepes pH 7.4. For 2D calcium switch, cells were seeded on sterilized coverslips and grown to 100% confluency. Cells were then rinsed 3x with PBS and incubated for 40-48 hours in SMEM (calcium free, 4.5 g/L glucose) supplemented with 5µM $CaCl_2$, 5% FBS dialyzed in PBS to remove Ca^{2+} and Mg^{2+}, and 20mM Hepes (pH 7.4). Calcium switch was triggered by changing media back to DMEM (1.8mM Ca^{2+}). For 3D cyst culture, single cells were diluted to $6.67x10^3$ cells/mL in media with 2% matrigel (BD Bioscience) and layered on 12-30 µL matrigel in 8-well coverslides (Biotek), on sterilized coverslips, or in 35mm glass-bottomed dishes (Mattek).

Antibodies and reagents

Antibodies used include: mouse anti-GP135 (1:25, from G. Ojakian, SUNY Downstate Medical Center), mouse anti-α tubulin (1:300, DM1A, Sigma), mouse anti-acetylated tubulin (1:200, Sigma), mouse anti-GM130 (1:100, BD Transduction), rabbit anti-detyrosinated tubulin (1:500, SG, from G. Gundersen, Columbia University), rabbit anti-pericentrin (1:500, Covance), rabbit anti-ninein (1:100, Bethyl Laboratories), rabbit anti-NEZHA (1:250, Sigma), rat anti-tyrosinated tubulin (1:50, YL1/2, from G. Gundersen, Columbia University), rat anti-E-Cadherin (1:50, Sigma), rat anti-EB1 (1:100, KT51, Santa Cruz), rat anti-ZO1 (1:100, Santa Cruz), human anti-Golgi and

TGN (1:50, G3 and G4, from A. Gonzalez Pontifica Universidad Catolica de Chile, Santiago, Chile), fluorescently conjugated secondary antibodies (varies, Jackson Immunoresearch). Other staining reagents include: Rhodamine-tagged phalloidin (1:200, Invitrogen), DAPI (1:10,000, Molecular Probes).

Cell fixation

2D calcium switched cells were rinsed with PBS and fixed at RT, unless otherwise stated, using the following methods: 4% PFA in PBS fixation for 15 minutes and 0.5% Triton-X in PBS permeabilization for 5 minutes, 0.5% Glutaraldehyde and 0.5% Triton-X in PEM buffer (100mM PIPES, 2mM EGTA, 2mM $MgCl_2$, pH 6.8) fixation/permeabilization for 20 minutes with 0.2% $NaBH_4$ in PBS quenching for 5 minutes x 2, Methanol fixation for 45-60 seconds at -20ºC. 3D cysts were rinsed with PBS and fixed using the following methods: 4% PFA in PBS fixation for 30 minutes and 0.5% Triton-X in PBS permeabilization for 15 minutes, 0.5% Glutaraldehyde and 0.5% Triton-X in PEM buffer fixation/permeabilization for 40 minutes with 0.2% $NaBH_4$ in PBS quenching for 10 minutes x 2, Methanol fixation for 2 minutes at -20ºC. 2D coverslips were stained for 30 minutes at RT, washed 1x with PBS at RT, and mounted in Prolong Gold (Life Technologies). Cysts were stained ON at 4ºC, washed 2x with PBS for 20 minutes at RT, and mounted in Fluoromount G (Southern Biotech).

Fixed and time-lapse imaging and analysis

Fixed coverslips were imaged on a Nikon TiE upright microscope with a 60x (NA 1.4) Plan-Apochromat oil immersion objective lens using epifluorescence or confocal (Crest Optics). Images were collected with digital charge-coupled device cameras: 6.45 µm pixels, 560MHz for 16-bit epifluorescence images (Neo sCMOS; Andor Technology), 6.45 µm pixels, 1 MHz for 14-bit confocal

images (ORCA II-ER; Hamamatsu Photonics). Devices were controlled with either Elements (Nikon) or Metamorph™ (Molecular Devices). All fixed images were acquired at 0.3 µm z intervals. Images were processed with nearest neighbors deconvolution (Metamorph) or 3D deconvolution (Autoquant). Representative images were scaled to 8-bit for figure assembly and are maximum projections of 3-5 planes through the cell. For time-lapse imaging, coverslips were transferred to recording media (Hank's balanced salt solution supplemented with 4.5 g/L glucose, 1% FBS, 20 mM Hepes pH 7.4, essential and nonessential amino acids) and placed in a temperature-controlled recording chamber at 36ºC (Harvard Apparatus). Images were acquired at 2 or 3 second intervals with a 40x phase objective. Images were processed using Metamorph to remove average fluorescence over time and highlight only moving objects. EB1 comets were analyzed qualitatively for directionality only if they were near VACs/lumens and remained moving in the field of view for 10 seconds.

Plasmids and transfection

EB1-eGFP was provided by Dr. Katsuhiro Kita (Weill Cornell Medical College) (created by Dr. Lynn Cassimeris (Lehigh University)) and mCherry-Actin was provided by Dr. Alan Hall (Memorial Sloan Kettering). Cells were transfected with 5µg of each DNA using nucleofection (Amaxa) as recommended by the manufacturer. Cells were seeded densely onto coverslips or sparsely into matrigel on sterilized round coverslips or in a 35mm glass-bottomed dish (Mattek).

Drug treatment and lumen formation quantification

For 2D experiments, cells were treated at the time of calcium switch with either 10µm taxol (Sigma) or 33µm nocodazole (GE Healthcare). Coverslips were

fixed at 2 hour intervals using PFA and stained for GP135 and nuclei. Two fields were imaged per coverslip and quantified for both the total number of cells and the localization of GP135 in each cell. For 3D experiments, media was replaced with DMEM containing 2% matrigel with or without 10µm taxol or 33µm nocodazole at exactly 24 hours after seeding. Cysts were fixed at 3 hour intervals using PFA and stained for GP135 and nuclei. Forty cysts containing between 2 and 4 cells in interphase were scored per timepoint and condition based on the localization of GP135. In cases where GP135 was localized differently in two cells in the same cyst, cysts were scored as the most advanced event. Each experiment was done three times. Statistical significance was determined by sequentially performing a two-way ANOVA test on the entire data set, which indicated that the data differed enough to test, then by Bonferroni's post-test between control and drug treated cells/cysts with lumens.

MT Growback

For 2D experiments, cells were calcium switched for 2 hours before transfer to DMEM (4.5 g/L glucose) without $NaHCO_3$ supplemented with 5% FBS, 10mM Hepes pH 7.2, and 33µM nocodazole. For 3D experiments, cysts on small round coverslips were transferred 24-33 hours after seeding to the same media supplemented with 2% matrigel. Cells were placed on ice for 1 hour and transferred back to 37°C for 2 hours. Coverslips were rinsed 4-6x in PBS at 37°C and transferred to media without nocodazole at 37°C for 10 minutes. Cells were permeablized with 0.5% Triton-X in PEM for 45 seconds (75 seconds for cysts) and rinsed 4-6x in PBS at 37°C. Cells were immediately fixed in 0.5% glutaraldehyde for 20 minutes at RT (cysts for 40 minutes). Coverslips were quenched and stained as previously described.

CHAPTER THREE

A MODEL FOR HOW MICROTUBULES AND KINESINS CONTRIBUTE TO APICAL LUMEN FORMATION

In this thesis project, I characterized how MTs are organized during early events in epithelial polarization and showed that perturbing the array at these stages either delays or inhibits lumen formation. Based on MT organization during lumen formation, I can speculate on how rho and par signaling affect the cytoskeleton during early polarization events. As was previously described, RhoA is downregulated and Rac1 upregulated at the ECM-facing membrane prior to GP135 endocytosis while Cdc42 localizes to the apical membrane initiation site (AMIS) before lumen formation (Martin-Belmonte, Yu et al. 2008; Yu, Shewan et al. 2008). Considering the known effects of these proteins on MTs in other systems, it seems likely that Cdc42 is mediating MT stabilization at the AMIS since modified MTs are localized near the lumen following its formation. Additionally, Cdc42 signals upstream to prevent deactivation of APC, which is required for both MT stabilization and polarization in epithelial cells (Etienne-Manneville and Hall 2003; Mimori-Kiyosue, Matsui et al. 2007). Since there are no modified MTs oriented towards the ECM-facing surface, Rac1 mediated stabilization is not likely. Additionally, since Par1 is segregated to the lateral surface by aPKC and is known to promote MAP dissociation from the MT lattice (Nakano and Takashima 2012), it is likely that MTs near the basolateral domain are more dynamic. Finally, RhoA mediated stabilization is not likely since modified MTs are only localized near the lumen during 2D calcium switch, even though RhoA is likely active at the reformed adherens junctions surrounding the entire periphery. To test which of the rho GTPases signal MT reorganization, the

modified MT distribution could be analyzed during lumen formation following rho knockdown or inhibition. Perturbing each rho GTPase may reveal which pathway induces MT stabilization, but may also cause too many polarization defects to interpret the results. Known effectors of MT stability downstream of rho GTPases could also be perturbed, such as mDia for RhoA, CLASP2 for Rac1, and aPKC for Cdc42, though, to date, these proteins have only been shown to affect MT stability in migrating cells and may not be active during lumen formation (Etienne-Manneville and Hall 2003; Wen, Eng et al. 2004; Drabek, van Ham et al. 2006). Whether they are active and provide analogous signals to promote formation of the apical membrane and lumen is not yet known.

Since the distribution of stable, modified MTs changes within the cell depending on whether the apical membrane is localized in VACs or in a lumen, stable MTs may be formed by growing MT plus end capture and could determine the site of lumen formation (Figure 10). There are two possible ways that this could occur, based on what is known about MT stabilization in epithelial and other cells. First, the formation of cell-cell adhesions likely stabilizes MTs. Since homotypic E-Cadherin interactions are sufficient to induce MT stabilization (Chausovsky, Bershadsky et al. 2000), it is likely that the first adhesions formed following cell division signal MT plus-end capture. Second, this may occur following the establishment of the AMIS, since it has been previously established that Cdc42 localizes to the AMIS and recruits Par3/Par6/aPKC prior to lumen formation (Martin-Belmonte, Gassama et al. 2007). In migrating cells, aPKC downregulates GSK3β signaling to promote APC interaction with MT plus ends (Etienne-Manneville and Hall 2003). Both of these sites could also recruit dynein, through β-catenin and Tctex-1 at

adherens junctions (Ligon, Karki et al. 2001) and through Par3 at the AMIS (Schmoranzer, Fawcett et al. 2009). In migrating cells, dynein provides a pulling force on stable MTs that maintains centrosome orientation during rearward nuclear movement (Gomes, Jani et al. 2005). During lumen formation, MT plus-end capture and dynein activity may provide a pulling force to reorient the centrosome and Golgi, which could reposition VACs near the site of lumen formation. This scenario is likely since lumen formation does not occur in MDCK cells unless the centrosome is localized between the nucleus and the AMIS, and this localization is dependent on aPKC, which is upstream of APC-mediated MT stabilization (Rodriguez-Fraticelli, Auzan et al. 2012). To test this, each of the described effectors in this pathway (E-Cadherin, β-catenin, Tctex-1, dynein, Cdc42, Par3) could be knocked down or inhibited to see whether they are required for lumen formation. Then this pathway can be elucidated by observing the distribution of modified MTs and the position of the centrosome in cells that fail to form lumens.

MT stabilization near the site of lumen formation may also orient trafficking routes for MT motors to transport the apical membrane. Previous work in our lab showed that Kif17, a kinesin-2 family motor, mediates MT stabilization via EB1 and APC and is also required for lumen formation in 3D cysts (Jaulin and Kreitzer 2010). Mature cysts have either no lumen or multiple lumens following Kif17 knockdown, indicating that GP135 transcytosis may still occur, though most likely after multiple rounds of cell division when cells have overcome the lack of either a targeting signal or trafficking machinery, or possible both. The modified MT distribution near the forming lumen suggests that Kif17/EB1/APC-mediated MT stabilization occurs near the lumen. Therefore, Kif17 may provide the initial targeting signal for lumen formation by

stabilizing MTs, or could be responsible for transport of the apical membrane from the VAC to the lateral membrane. This requires a more in-depth analysis of lumen formation following Kif17 knockdown in cysts or during calcium switch, paying specific attention to both the modified MT distribution following GP135 endocytosis and apical membrane trafficking from the VAC to the lumen. If Kif17does not mediate transport of the apical membrane, other candidates can be knocked down or inhibited to see whether lumen formation is able to occur. Since Rab3A is required for lumen formation and localized to VACs (Galvez-Santisteban, Rodriguez-Fraticelli et al. 2012), Kinesin-3 family members may mediate transport since Kif1A and Kif1Bβ have been shown to interact with Rab3A in neurons (Niwa, Tanaka et al. 2008). Alternatively, if stabilized MTs are providing tracks from the VAC to the site of lumen formation, Kif5B may mediate transport since it is known to preferentially interact with modified MTs and transport an apical cargo (Liao and Gundersen 1998; Reed, Cai et al. 2006; Jaulin, Xue et al. 2007).

In this thesis, I've confirmed that MTs are required for lumen formation in MDCK cells, which has been speculated but not formally tested, and have hypothesized why this is based on the structural aspects of the array. Additionally, the distribution of modified MTs and centrosome location during GP135 transcytosis in 2D and 3D indicates how proteins that regulate epithelial polarity also signal MT reorganization and stabilization. In future directions, the aspects of this MT array can be used as a readout for how polarization signals influence MT reorganization. Perturbing known polarity regulators should reveal the upstream pathways contributing to MT stabilization and reorganization, while perturbing MT motors should reveal whether MT tracks are required for transport of the nascent apical membrane.

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